TRYPANOSOMA EVANSI IN NORTHERN ETHIOPIA: EPIDEMIOLOGY, DIVERSITY AND ALTERNATIVE DIAGNOSTICS

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List of abbreviations

AAT	animal African trypanosomosis
AFLP	amplified fragment length polymorphism
aqp2/3	aquaglyceroporin 2/3
ATP	adenosine tri-phosphate
bp	base pair
BSA	bovine serum albumin
BSF	bloodstream form
bw	body weight
CATT	Card Agglutination Test for Trypanosomiasis
CDS	coding sequence
CI	confidence interval
CNS	central nervous system
DA	diminazene aceturate
DAPI	4',6-diamidino-2-phenylindole
DEAE	di-ethyl-amino-ethyl
Dk	dyskineplastic
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DPI	days post infection
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
GDP	gross domestic product
GEB	guanidine EDTA buffer
GPI	glycosylphosphatidylinositol
HAPT1	high affinity pentamidine transporter 1
HAT	human African trypanosomosis
HMI-9	Hirumi's modified Iscove's medium 9
HS	horse serum
IC_{50}	50% inhibitory concentration
ICT	immunochromatographic test
IFAT	indirect fluorescence antibody test
IFN	interferon
Ig	immunoglobulin
IL	Interleukin
IM	intramuscular

IP	intraperitoneal
ISG	invariant surface glycoprotein
ISM	Isomethamidium chloride
ISSR	inter-simple sequence repeats
ITM	Institute of Tropical Medicine
ITS	internal transcribed spacer
IV	intravenous
K	kappa
Kb	kilobase
kDa	kilodalton
kDNA	kinetoplast deoxyribonucleic acid
kg	kilogram
LAMP	loop-mediated isothermal amplification
LB	Luria Bertani
LDL	low density lipoprotein
m.a.s.l.	meter above sea level
mAECT	mini-Anion Exchange Centrifugation Technique
MAP	microtubule associated proteins
Mb	mega base
mHCT	micro haematocrit centrifugation technique
Nbs	nanobodies
NO	nitirc oxide
N-terminal	amino terminal
NTS	non-transcribed spacer
NTTAT	non-tsetse transmitted animal trypanosomoses
OIE	Office International des Epizooties
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PSG	phosphate buffered saline glucose buffer
Q-PCR	quantitative polymerase chain reaction
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RDT	rapid diagnostic test
RFLP	restriction fragment length polymorphism
RIME	random insertion mobile element
RNA	ribonucleic acid
RoTat	Rode *Trypanozoon* antigen type
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid

SC	subcutaneous
SNP	single nucleotide polymorphism
SrRNA	small ribosomal ribonucleic acid
SSA	sub Saharan Africa
SSU	small subunit
STIB	Swiss Tropical Institute Basel
T.	*Trypanosoma*
T.b.	*Trypanosoma brucei*
Taq	*Thermus aquaticus*
T_d	doubling time
*Tev*AT1	*T. evansi* adenosine transporter 1
TL	immune trypanolysis test
TNF	tumour necrosis factor
TTAT	tsetse transmmited animal trypanosomosis
TU	transcribed unit
*Tv*PRAC	*Trypanosoma vivax* proline racemase
VAT	variable antigenic type
VSG	variant surface glycoprotein

Summary

Animal African trypanosomosis (AAT) is a complex of parasitic diseases of various domestic and wild animal species caused by different species of trypanosomes. *Trypanosoma (T.) brucei, T. congolense* and *T. vivax* are transmitted by tsetse flies.

Trypanosoma evansi, but also *T. vivax*, is mechanically transmitted by other biting flies and *T. equiperdum* is sexually transmitted in *Equidae*. All these pathogenic trypanosome species occur in Ethiopia. In particular, surra caused by *T. evansi*, is the number one parasitic disease of camel that is the main domestic animal species in many pastoral communities and that may become increasingly important with the current climate change. AAT entails serious economic losses due to mortality, morbidity and reduction in productivity. Compared to tsetse-transmitted AAT, the attention given towards control and research on non-tsetse transmitted animal trypanosomoses (NTTAT) is negligible. This doctoral study can be seen as a contribution to increase our knowledge on NTTAT due to *T. evansi* and to attract the attention of policy makers and the international research community for this disease.

The study, conducted partly in Ethiopia and partly in Belgium, aimed at 1° defining the epidemiological situation of NTTAT in domestic animals in Tigray and Afar regions in Northern Ethiopia, 2° isolating trypanosomes from infected animals, 3° improving the molecular and serological diagnosis of surra.

A cross-sectional epidemiological survey was conducted on 754 dromedary camels, 493 cattle, 264 goats, 181 sheep, 84 donkeys, 25 horses and 10 mules. Overall parasitologically confirmed prevalence of NTTAT was 3.8% (68 animals) and was significantly higher in cattle (7.3%) than in camels (4.0%), sheep (0.6%) and goats (0.4%). No trypanosomes were detected in equines. Buffy coat samples from parasitologically positive animals were cryostabilised in a special cryomedium for subsequent isolation. Antibody detection with CATT/*T. evansi* revealed an overall seroprevalence of 19.6% with significantly higher seroprevalence in cattle (37.3%) than in camels (13.7%), goats (13.3%), sheep (12.7%) and donkeys (10.7%). These high seroprevalences could not be confirmed in the immune trypanolysis test (TL) which is considered fully specific for *T. evansi*. Only part of this discrepancy between both antibody detection tests can be attributed to the presence of *T. vivax* in the studied animals. The latter species was detected by the TvPRAC PCR in 3.5% of the camels, 3.0% of the goats, 2.6% of the cattle and 2.2% of the sheep but not in equines. Two camels and one goat harboured a mixed infection with *T. evansi* and *T. vivax*. Overall molecular prevalence of *T. evansi* type A, assessed with RoTat 1.2 PCR, was 8.0% and was significantly higher in horses (28.0%), mules (10.0%) and camels (11.7%) than in cattle (6.1%), donkeys (6.0%), goats (3.8%) and sheep (2.2%). Four camels, all from Awash Fentale district in Afar, were positive in the *T. evansi t*ype B specific EVAB PCR thus providing the first molecular evidence of *T. evansi* type B in Northern Ethiopia. All four were negative in CATT/*T. evansi* and TL although one of them was also positive in RoTat 1.2 PCR suggesting a

mixed infection. The higher serological prevalence as compared to the molecular prevalence of *T. evansi*, particularly in ruminants, could be explained by the fact that antibody detection tests like CATT/*T. evansi*, cannot distinguish current from cured infection and that during chronic infections, parasitaemia can be far below the detection limit of parasitological and molecular tests. Also, the CATT/*T. evansi* can cross-react with other infections.

Among the 68 parasitologically positive animals, 34 were negative in *T. evansi* and *T. vivax* specific PCRs and were checked with ITS1-PCR for the possibility of infections with *T. theileri* and *T. congolense*. Two bovine were positive for *T. theileri* and no animal was positive for *T. congolense*.

The isolation of trypanosomes from the 68 parasitologically positive buffy coat samples from 36 cattle, 30 camels, 1 sheep and 1 goat was conducted in immunosuppressed mice and yielded 22 *T. evansi* stocks, all from camels. Not surprisingly, no *T. vivax* stocks could be isolated in the mouse model. Typing by PCR on the original buffy coats revealed 20 *T. evansi* type A (positive in RoTat 1.2 PCR) and 2 *T. evansi* type B (positive in EVAB PCR). Twelve of the type A stocks and both type B stocks were brought to Belgium for further investigation, included adaptation to *in vitro* culture for *in vitro* drug sensitivity testing. After *in vivo* expansion, and re-typing, nine stocks were confirmed as type A, two as type B and three stocks appeared to be mixed infections with both types. One *T. evansi* type A stock was akinetoplastic, i.e. had lost its mitochondrial DNA consisting of concatenated circular DNA densily packed in an organelle called kinetoplast. While expansion in mice allowed to propagate the mixed infections, *in vitro* culture was selective for *T. evansi* type B. Furthermore, multiple *in vitro* passages induced the loss of the kinetoplast in some stocks but infectivity to mice was not affected. *In vitro* drug sensitivity assays with melarsomine dihydrochloride, diminazene diaceturate, isometamidium chloride and suramin revealed no resistance against these trypanocidal drugs in the five *in vitro* adapted stock from Northern Ethiopia. In order to address some limitations of the current molecular tests for typing *T. evansi*, the gene of the F1-ATP synthase γ subunit of eight Northern Ethiopian *T. evansi* stocks and some other reference strains was sequenced. Type-specific single nucleotide polymorphisms (SNPs) and deletions observed within this gene, may provide new markers to identify the *T. evansi* type that do not rely on variant surface glycoprotein, genes or kinetoplast DNA. In addition, MORF-2 REP analysis indicated two distinct allelic profiles in *T. evansi* type A stocks and that they are different from the Indonesian RoTat 1.2 reference strain. The MORF-2 REP allelic profiles showed that the Northern Ethiopian *T. evansi* type B stocks are distinct from the Kenyan *T. evansi* type B.

Control of AAT relies on detection of infected animals followed by administration of trypanocidal drugs. In routine practice, diagnosis of surra is limited to the observation of unspecific clinical signs. If at all applied, parasitological techniques that are commonly used for the diagnosis of surra have limited sensitivity and molecular diagnostics are simply not adapted for routine diagnosis in developing countries. Therefore, serodiagnosis by means of detection of *T. evansi*-specific antibodies, for example with the Card Agglutination Test for *T. evansi* (CATT/*T.*

evansi), ELISA or immune trypanolysis (TL), is recommended by the World Organization for Animal Health (OIE). Among these test, only CATT/*T. evansi* can be applied in the field although it is still dependent on electricity to run the rotator and to respect the cold chain needed to preserve the quality of the antigen. As such, CATT/*T. evansi* does not fully comply with the ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment-free and delivered) criteria of a diagnostic test required in the 21st century. Moreover, it is produced with native antigens purified from trypanosomes grown in laboratory animals. Recently, an alternative antibody detection test for serodiagnosis of *T. evansi* infection, the Surra Sero *K*-SeT, was developed by ITM and Coris BioConcept, a Belgian diagnostic company. Surra Sero *K*-SeT is an immunochromatographic test (ICT) where the antigen consists of an N-terminal fragment of RoTat 1.2 VSG, recombinantly expressed in *Pichia pastoris*. In this doctoral study, we compared the diagnostic accuracy of Surra Sero *K*-SeT and CATT/*T. evansi* with TL as reference test by testing sera from 300 camels, 100 water buffaloes, 100 horses, 82 bovines, 88 sheep, 99 dogs and 37 alpacas. The Surra Sero *K*-SeT displayed considerably higher sensitivity than CATT/*T. evansi* (98.1% *versus* 84.4%) but somewhat lower specificity (94.8% *versus* 98.3%). In particular and for unknown reasons, the specificity with the alpaca sera was disappointingly low (83.8%). Unfortunately, we were not able to test the Surra Sero *K*-SeT on sera from camels infected with *T. evansi* type B but we hypothesize that it cannot detect type B infections thus jeopardising its diagnostic potential in countries where *T. evansi* type B is present, like Kenya, Ethiopia and possibly Sudan.

In conclusion, this doctoral study revealed that, in terms of prevalence, NTTAT due to *T. evansi* type A and type B and *T. vivax*, is an important threat to animal health in Tigray and Afar and not only in camel and cattle but also in small ruminants and equines. Control of AAT, in Ethiopia and elsewhere, should therefore not only focus on tsetse transmitted trypanosomes and should take into consideration the role of small ruminants and equines in the epidemiology of the disease. This study allowed us to establish an important new collection of *T. evansi* stocks from Northern Ethiopia, including, two *T. evansi* type B stocks. Genetic characterization of these stocks may eventually lead to an improved genetic marker for type B, based on SNPs in the F1-ATP γ–subunit gene. In order to adapt the Surra Sero *K*-SeT so that it can detect *T. evansi* type B infections, other candidate invariable antigens and other expression systems should be investigated.

Samenvatting

Dierlijke Afrikaanse trypanosomosis (AAT) is een verzameling van parasitaire infecties bij diverse gedomesticeerde en wilde dieren, veroorzaakt door verschillende soorten trypanosomen. *Trypanosoma* (*T.*) *brucei*, *T. congolense* en *T. vivax* worden overgebracht door tseetsee vliegen. *Trypanosoma evansi*, maar ook *T. vivax*, worden mechanisch overgebracht door steekvliegen en *T. equiperdum* is een sexueel overdraagbaar in Equidae. Al deze pathogene trypanosomen komen voor in Ethiopië. Vooral surra, veroorzaakt door *T. evansi*, is de meest voorkomende parasitaire aandoening in de dromedaris die een zeer belangrijke gedomisticeerde soort is voor herder gemeenschappen en die, in het licht van de huidige klimaatveranderingen, steeds belangrijker wordt. AAT is verantwoordelijk voor grote economische verliezen als gevolg van mortaliteit, morbiditeit en productiviteitsverlies. Vergeleken met tseetsee-overgedragen AAT wordt weinig aandacht besteed aan niet-tseetsee-overgedragen dierlijke trypanosomosis (NTTAT). Met deze doctoraatsthesis willen we bijdragen tot de kennis van NTTAT veroorzaakt door *T. evansi* en willen we deze ziekte onder de aandacht brengen van beleidsmakers en de internationale wetenschappelijke gemeenschap.

Deze studie, ten dele uitgevoerd in Ethipië en ten dele in België, beoogde 1° de epidemiologie van NTTAT in gedomesticeerde dieren in Tigray en Afar in noordelijk Ethiopië te kennen; 2° trypanosomen te isoleren van geïnfecteerde dieren; 3° de moleculaire en serologische diagnose van surra te verbeteren.

Een cross-sectionele epidemiologische survey werd uitgevoerd op 754 dromedarissen, 493 runderen, 264 geiten, 181 schapen, 84 ezels, 25 paarden en 10 muildieren. De algemene parasitologische prevalentie van NTTAT was 3.8% (68 dieren) en was significant hoger in runderen (7.3%) dan in dromedarissen (4.0%), schapen (0.6%) en geiten (0.4%). Bij geen enkele paardachtige werden trypanosomen gevonden. Buffy coat stalen van parasitologisch positieve dieren werden gecryppreserveerd op vloeibare stikstof in een speciaal cryomedium voor de isolatie van de trypanosomen achteraf. Antistof detectie met CATT/*T. evansi* toonde een algemene seroprevalentie van 19.6% met significant hogere seroprevalentie in runderen (37.3%) dan in dromedarissen (13.7%), geiten (13.3%), schapen (12.7%) en ezels (10.7%). Deze hoge prevalenties konden echter niet bevestigd worden in immune trypanolyse (TL) die als absoluut specifiek wordt beschouwd voor *T. evansi* antistoffen. De discrepantie tussen beide antistof detectie tests kan slechts gedeeltelijk toegeschreven worden aan infectie met *T. vivax* in de onderzochte dieren. Deze trypanosoom soort werd met behulp van TvPRAC PCR aangetoond in 3.5% van de dromedarissen, 3.0% geiten, 2.6% runderen and 2.2% schapen maar niet in de paardachtigen. Twee dromedarissen en één geit vertoonden menginfecties van *T. evansi* en *T. vivax*. De algemene moleculaire prevalentie van *T. evansi* type A, gemeten met de RoTat 1.2 PCR, was 8.0% en was significant hoger in paarden (28%), muildieren (10%) en dromedarissen (11.7% dan in runderen (6.1%), ezels (6.0%), geiten (3.8%) en schapen (2.2%). Vier dromedarissen, allemaal van Awash Fentale disctrict, waren positief voor *T. evansi* type B in de EVAB PCR.

Daarmee toonden we voor de eerste keer via moleculaire diagnose aan dat *T. evansi* type B ook in noordelijk Ethiopië voorkomt. Deze vier dromedarissen waren allen negatief in CATT/*T. evansi* en TL alhoewel één ervan ook positief was voor RoTat 1.2 PCR wat wijst op een menginfectie. De hogere seroprevalentie in vergelijking met moleculaire prevalentie van *T. evansi*, in het bijzonder in de runderen, kan verklaard worden door het feit dat antistof tests zoals CATT/*T. evansi* geen onderscheid kunnen maken tussen actieve en genezen infectie en dat in chronische infecties de parasitemie ver beneden de detectielimiet van parasitologische en moleculare diagnostische tests ligt. Ook is het geweten dat CATT/*T. evansi* kan kruisreageren met andere infecties. Onder de 68 parasitologisch positieve dieren waren er 34 negatief in *T. evansi* en *T. vivax* specifieke PCRs. Deze werden getest met ITS1-PCR om mogelijke infecties met *T. theileri* en *T. congolense* aan te tonen. Twee runderen waren positief voor *T. theileri* terwijl geen enkel dier positief was voor *T. congolense*.

De isolatie van trypanosomen uit de 68 parasitologisch positieve buffy coat stalen van 36 runderen, 30 dromedarissen, 1 schaap en 1 geit gebeurde door inoculatie van geïmmunosupprimeerde muizen en leverde 22 *T. evansi* stammen op, enkel van dromedarissen. Niet onverwacht werd geen enkele *T. vivax* stam geïsoleerd in het muismodel. PCR op de oorspronkelijke buffy coat stalen toonde twintig *T. evansi* type A (positief in RoTat 1.2 PCR) en twee *T. evansi* type B (positief in EVAB PCR). Twaalf van de type A stammen en beide type B stammen werden naar België gebracht voor verder onderzoek, inbegrepen het aanpassen aan *in vitro* cultuur voor *in vitro* drug gevoeligheid tests. Na *in vivo* expansie en hertypering werden negen stammen geconfirmeerd als type A, twee als type B en drie stammen bleken gemengde infecties te zijn van type A en B. Eén *T. evansi* type A stam was akinetoplast d.w.z. heeft zijn mitochondriaal DNA verloren dat bestaat uit aan elkaar geklonken circulaire DNA strengen die dicht opeen gepakt zijn in een organel dat kinetoplast wordt genoemd. Waar expansie in muizen de gemengde infecties in stand hield blijken *in vitro* culturen selectief te zijn voor *T. evansi* type B. Verder blijkt dat herhaaldelijke *in vitro* passages leidden tot het verlies van de kinetoplast in sommige stammen maar niet tot verminderde infectiviteit voor muizen. *In vitro* drug gevoeligheidstests met melarsomine dihydrochloride, diminazene diaceturate, isometamidium chloride en suramine konden geen resistentie aantonen tegen deze medicamenten in de vijf geteste *T. evansi* stammen van noordelijk Ethiopië. Om een aantal beperkingen van de bestaande moleculaire tests voor *T. evansi* typering te overkomen werd het F1-ATP synthase γ subunit gen van acht *T. evansi* stammen uit noordelijk Ethiopië en van enkele andere referentie stammen gesekweneerd. In dit gen werden type-specifieke "single nucleotide polymorphisms" (SNPs) en deleties waargenomen die nieuwe merkers kunnen opleveren om het *T. evansi* type te identificeren, onafhankelijk van variabele oppervlakte eiwit genen of van kinetoplast DNA. Bovendien toonde MORF-2 REP analyse het bestaan aan van twee verschillende allelische profielen in *T. evansi* type A stammen die verschillen van de Indonesische RoTat 1.2 referentie stam. Deze MORF-2 REP allelische profielen toonden ook aan dat de noord Ethiopische *T. evansi* type B stammen verschillend zijn van de *T. evansi* type B stam uit Kenia.

Controle van AAT berust op detectie van geïnfecteerde dieren gevolgd door behandeling. In de routine praktijk blijft diagnose van surra beperkt tot het herkennen van aspecifieke symptomen. Parasitologische technieken, als ze al toegepast worden, hebben meestal een beperkt gevoeligheid en moleculaire diagnostica zijn eenvoudigweg niet geschikt voor routine toepassing in endemische landen. Daarom beveelt de Wereld Organisatie voor Dierenwelzijn (OIE) serodiagnose aan op basis van het aantonen van *T. evansi* specifieke antistoffen. Voorbeelden zijn de Card Agglutination Test for *T. evansi* (CATT/*T. evansi*), ELISA en immuno trypanolyse. Van deze tests is enkel de CATT/*T. evansi* toepasbaar in het veld alhoewel ook die nog afhankelijk is van elektriciteit om de rotator aan te drijven en om de koude keten te handhaven. Daarme voldoet de CATT/*T. evansi* niet volledig aan de ASSURED (affordable, sensitive, specific, user-friently, rapid, equipment-free and deliverable) criteria, vereist voor diagnostica van de 21ste eeuw. Bovendien wordt deze test geproduceerd met natieve antigenen, gezuiverd uit trypanosomen die worden opgegroeid in laboratoriumdieren. Recent werd een alternatieve antistof detectie test, de Surra Sero *K*-SeT, ontwikkeld door het ITG en Coris BioConcenpt, een Belgische firma. De Surra Sero *K*-SeT is een immunochromatografische test (ICT) waarin het antigeen bestaat uit een N-terminaal fragment van RoTat 1.2 VSG, recombinant tot expressie gebracht in *Pichia pastoris*. In dit doctoraatsonderzoek hebben we de diagnostische accuraatheid van de Surra Sero *K*-SeT vergeleken met CATT/*T. evansi* en met TL als referentietest. Deze vergelijking werd uitgevoerd op serumstalen van 300 dromedarissen, 100 waterbuffels, 100 paarden, 82 runderen, 88 schapen, 99 honden en 37 alpacas. De Surra Sero *K*-SeT vertoonde een duidelijk hogere gevoeligheid dan de CATT/*T. evansi* (98.1% *versus* 84.4%) maar een ietwat lagere specificiteit (94.8% *versus* 98.3%). Om tot nu toe onbekende redenen, was de specificiteit op alpacas ontmoedigend laag (83.8%). Spijtig genoeg konden we de Surra Sero *K*-SeT niet testen op dromedarissen geïnfecteerd met *T. evansi* type B maar we veronderstellen dat deze test geen type B infecties kan detecteren wat het diagnostisch potentieel ervan in landen zoals Kenia, Ethiopië en mogelijks Soedan, waar *T. evansi* type B voorkomt, compromiteert.

We besluiten dat deze doctoraatsstudie aantoont dat, in termen van prevalentie, NTTAT veroorzaakt door *T. evansi* type A en type B en door *T. vivax* een belangrijke bedreiging vormt voor dierengezondheid in Tigray en Afar en dit niet enkel voor dromedarissen en runderen maar ook voor kleine herkauwers en paardachtigen. Controle van AAT, in Ethiopië en elders, mag daarom niet enkel gericht zijn op tseetsee overdraagbare trypanosomen en moet rekening houden met de rol van kleine herkauwers en paardachtigen in de epidemiologie van de ziekte. Deze studie liet ons toe een belangrijke nieuwe collectie van *T. evansi* uit noordelijk Ethiopië uit te bouwen waaronder twee *T. evansi* type B stammen. Genetische karakterisatie van deze stammen kan eventueel leiden tot betere genetische merkers voor type B, gebaseerd op SNPs in het F1-ATP γ-subunit gen. Voor de aanpassing van de Surra Sero *K*-SeT zodat ook *T. evansi* type B infecties kunnen opgespoord worden, zal moeten gezocht worden naar kandidaat niet-variabele antigenen en een alternatief expressiesysteem voor hun recombinante productie.

Introduction

1. General introduction

Africa, with the highest population growth rates, faces serious challenges in feeding its population. About 233 million (20%) of people in the region are undernourished, with 31% of them in eastern Africa (FAO *et al.* 2015). The continent has about 300 million heads of cattle, 630 million sheep and goats, 140 million camels and 1.8 billion chicken and birds that play an important role in the life of rural and urban communities. The livestock sector contributes to 30 – 50% of the total agricultural Gross Domestic Product (GDP) in some African countries and plays a key role as livelihood asset (Hassane 2013). Half of the estimated 300 million poor people who live on less than USD 1.0 per day in sub Saharan Africa (SSA) are highly dependent on livestock. The role of livestock in food security and nutrition is through providing meat, milk, draught power, manure, fiber etc. Other livestock by-products such as wool, hides and skins add more economic value to the sector, which is valued to USD 14 billion per year of which, USD 9 billion is in the form of meat, milk and leather while USD 5 billion is in the form of organic fertilizer and draft power (AU-IBAR 2010).

The rapid human population increase, income growth and urbanization in SSA is believed to increase the demand for livestock products (Thornton 2010). However, the livestock sector faces various challenges that hinder it from meeting these expectations and that limit economic growth in this sector. It is principally affected by deficiencies in high productive breeds, food and water resources, animal health systems and disease control measures and service delivery, value addition, market information and market infrastructure, competitiveness and compliance with sanitary and phytosanitary standards. These are coupled with deficiencies in policy, legislative and institutional frameworks as well as with inadequate application of available technologies, knowledge and skills (AU-IBAR 2014). Among others, African trypanosomosis which affects people and livestock, is the major bottle neck of Africa's struggle against poverty which threatens human and livestock health and agricultural production, and, thereby, rural development and poverty alleviation in SSA (FAO 2014).

Tsetse and mechanically transmitted animal African trypanosomosis (AAT) is one of the main constraints to sustainable development of livestock farming in SSA, where the impact is manifested in disease burden, increased level of poverty, expenditure on controlling the disease, restricted access to fertile and cultivable areas, imbalances in land use and exploitation of natural resources and compromised growth and diversification of crop-livestock production systems (Shaw *et al.* 2013; Tesfaye *et al.* 2012; Mattioli *et al.* 2004). The main pathogenic African trypanosomes belong to three subgenera of the *Salivaria* section, namely, *Nannomonas* (*Trypanosoma (T.) congolense*), *Duttonella* (*T. vivax*), and *Trypanozoon*. The *Glossina* (tsetse fly) is responsible for tsetse-transmitted trypanosomosis ('nagana') due to *T. congolense, T. vivax* and *T. brucei* in 10 million square kilometers of Africa (Hoare 1972). Non-tsetse transmitted animal trypanosomoses (NTTAT) is caused by *T. evansi*, *T. equiprdum* and *T. vivax* infection. NTTAT due

to *T. evansi* and *T. vivax* is transmitted by biting flies, tabanids and *Stomoxys*, while *T. equiperdum* is a sexually transmitted disease of equines (Touratier 2000; OIE 2013b).

Trypanosomosis due to *T. evansi* (surra) is the number one disease of camels. However, horses are also very sensitive to this infection. Infected camels and equines may die within three months. Moreover, cattle, buffalo, pigs, goat and sheep suffer from immunosuppression, resulting in increased susceptibility to other diseases or vaccination failure (Gutiérrez *et al.* 2006a; Holland *et al.* 2003; Holland *et al.* 2001). The disease occurs in Africa, Asia, Latin America and with sporadic import cases in Europe (Desquesnes *et al.* 2013b; Gutiérrez *et al.* 2010).

Surra control is of great concern in order to protect the worldwide livestock production. Vaccination against the disease is unavailable; moreover, the insect vectors and animal reservoirs are still abundant. As a result, control programs mostly depend on accurate detection and treatment of infected cases (Desquesnes *et al.* 2013a; Nguyen *et al.* 2014). Currently, the treatments available for AAT are not species specific. However, correct diagnosis is a prerequisite for understanding the epidemiology and designing and implementation of sound control strategies (Pillay *et al.* 2013).

Diagnosis of a *T. evansi* infection usually starts with clinical symptoms or the detection of antibodies to *T. evansi*. However, conclusive evidence of *T. evansi* infection relies on detection of the parasite in the blood of infected animals. Unfortunately, parasitological techniques cannot always detect ongoing infections as the level of parasitaemia is often low and fluctuating, particularly during the chronic stage of the disease (Büscher 2014). The most sensitive parasitological test for trypanosomes of the *Trypanozoon* group is the mini-Anion Exchange Centrifugation technique (mAECT) with an analytical sensitivity of < 50 parasites per ml (Büscher *et al.* 2009). As an alternative to parasitological tests, a number of DNA detection tests such as PCR, Q-PCR and LAMP have been developed. The most sensitive are not *T. evansi* specific but will detect also *T. brucei* and *T. equiperdum*. Only few tests are claimed to be specific for *T. evansi*, including the PCR-RoTat 1.2 and Q-PCR RoTat 1.2 (Konnai *et al.* 2009; Claes *et al.* 2004). These molecular diagnostic tests are highly appreciated for surveillance and research purposes. However, since none of them are conceived as point-of-care tests, their value for diagnosis in rural settings where surra prevails is jeopardized.

For the detection of antibodies, the only test that is recommended by the World Animal Health Organisation is the CATT/*T. evansi* (OIE 2012). This test uses a *T. evansi* specific native purified variant surface glycoprotein (VSG) as antigen (*in casu* RoTat 1.2) (Bajyana Songa & Hamers 1988). The same antigen is also used in other test formats like the LATEX/*T. evansi* and ELISA/*T. evansi* but requires mass culture of *T. evansi* in rats (Verloo *et al.* 2000). The use of larger protein molecules in antibody detection tests gives rise to a number of false positives due to cross-reactivity with non *T. evansi* specific antibodies resulting in decreased test specificity (Büscher 2014). In addition, it has been found that diagnostic tests targeting the RoTat 1.2 VSG do not detect infection due to *T. evansi* type B (Ngaira *et al.* 2005). To avoid the use of laboratory

rodents for the production of native VSG Rode Trypanozoon antigen type 1.2 (RoTat 1.2), a recombinant antigen has been developed and used as antigen in ELISA and in latex agglutination (Lejon *et al.* 2005; Rogé *et al.* 2014; Rogé *et al.* 2013; Urakawa *et al.* 2001). None of the above mentioned serological test formats complies with the ASSURED criteria of diagnostic tests (affordable, sensitive, specific, user-friendly, rapid, equipment-free and delivered) (Mabey *et al.* 2004). A way to overcome this is to develop highly specific recombinant antigens that can detect infections due to *T. evansi* type A and B and that eventually will be incorporated into a rapid diagnostic test (RDT) for surra, which is designed without the need for host species specific conjugates.

2. Taxonomy of trypanosomes

Trypanosomes are unicellular flagelatted eukaryotes that belong to the order *Kinetoplastida*, suborder *Trypanosomatina* and family of *Trypanosomatidae*. On the basis of their invertebrate cycle and preferred host species, mammalian trypanosomes are divided into two major groups, the Stercoraria and Salivaria (Hoare 1972). The Stercoraria contain species in which the entire development is confined to the gut of the vector and infective metatrypanosomes can be found in the faeces of the insect. *T. cruzi,* the pathogenic trypanosome causing Chagas disease in Latin America, and *T. theileri,* which is a non-pathogenic parasite in bovine and buffaloes, are classical examples of stecorarians (Figure 1.1) (Rodrigues *et al.* 2006; Momen 1999).

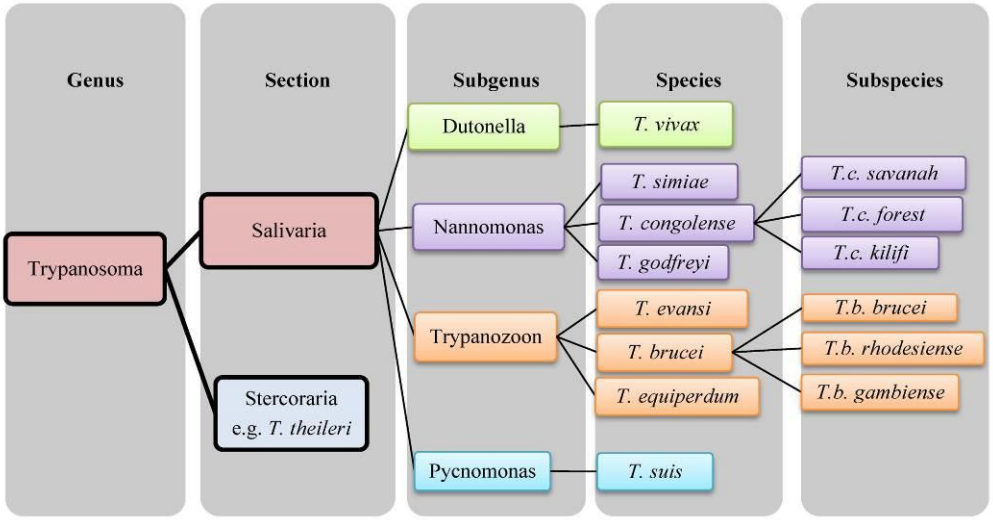

Figure 1.1: Schematic representation of the taxonomy of trypanosomes. Adapted from Gibson (2003).

Except *T. evansi* and *T. equiperdum* which do not have insect forms, Salivarian trypanosomes complete their cyclical development in the 'anterior station' of the vector and infective stages are transmitted to the mammalian host through the bite of an infected fly (Gibson & Bailey 2003).

3. Morphology and genetic diversity of *Trypanosoma evansi*

T. evansi the causative agent of surra, belongs to the genus *Trypanosoma*, subgenus *Trypanozoon* together with *T. brucei* (*b.*) *brucei*, *T. b. rhodesiense* and *T. b. gambiense* and *T. equiperdum* which cause nagana, human African trypanosomiasis (HAT) and the sexually transmitted disease of horses (dourine) respectively (Hoare 1972). *T. evansi* shares some characteristics with the other taxa of the subgenus *Trypanozoon*, such as the nucleic DNA, morphology and morphometry of the blood stage parasite. The slender forms are characterized by a thin posterior extremity, a large undulating membrane, a free flagellum, a spindle shaped cell, a central nucleus and a small subterminal kinetoplast (Figure 1.2 and 1.3) (Desquesnes *et al.* 2013b; Lai *et al.* 2008; Vickerman 1974).

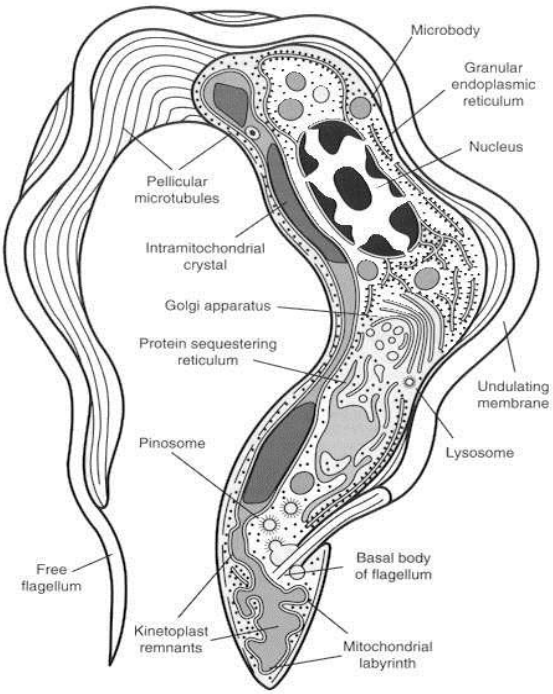

Figure 1.2: Fine structure of *T. evansi*, as revealed by transmission electron microcopy of thin sections (Vickerman 1974).

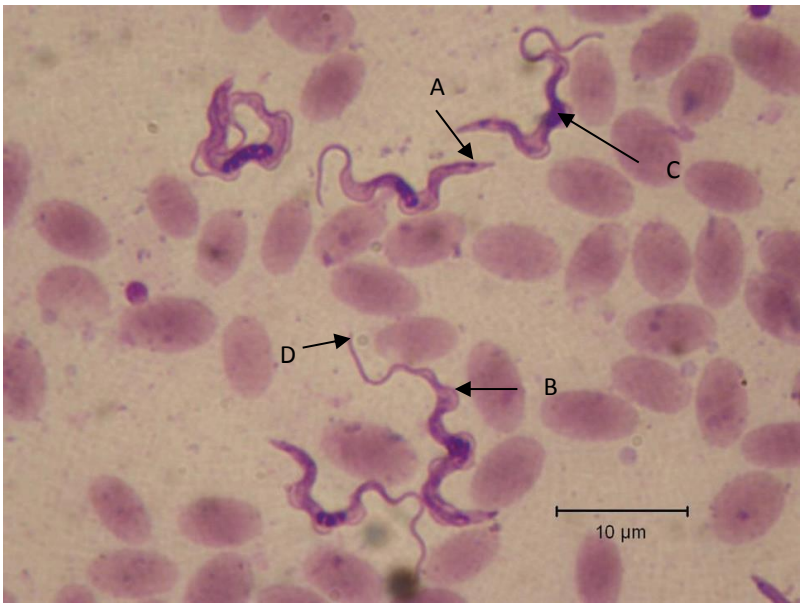

Figure 1.3: Morphological features of *T. evansi* in camel Giemsa stained blood smear: large size (25–35 µm), small and subterminal kinetoplast (A), thin posterior extremity, large undulating membrance (B), central nucleus (C), and free flagellum (D) (Desquesnes *et al.* 2013b).

The kinetoplast corresponds with the DNA (kDNA) of the unique mitochondrion of trypanosomatids. This kDNA consists of a huge network of interlocked circular DNA molecules of two types: maxicircles and minicircles (Lukes *et al.* 2005). The maxicircle with a size of ±23-kb in 20–50 copies, contains a typical set of rRNA and protein-coding genes, most of which encode subunits of respiratory chain complexes. The minicircle kDNA comprise a highly diverse set of thousands of ±1-kb minicircles, which encode guide RNAs required for posttranscriptional editing (Schnaufer *et al.* 2002; Stuart *et al.* 1997; Fidalgo & Gille 2011).

T. equiperdum and *T. evansi* are dyskinetoplastic (kDNA^{-}) since they lack part of the kDNA (Claes *et al.* 2005; Lai *et al.* 2008; Schnaufer *et al.* 2002; Carnes *et al.* 2015). *T. equiperdum* typically has retained maxicircles, in some cases with substantial deletions, but has lost its minicircle diversity. *T. evansi* does not have maxicircles and either shows minicircle homogeneity or are akinetoplastic (kDNA°) (Ou *et al.* 1991; Lun & Vickerman 1991; Ventura *et al.* 2000; Schnaufer *et al.* 2002).

T. evansi is biochemically similar to its ancestor *T. b. brucei* but it is no longer able to undergo a cycle in *Glossina* due to the loss of the maxicircle kinetoplast DNA (kDNA), hence its inability to perform oxidative phosphorylation (Hoare 1972; Borst *et al.* 1987; Lun & Desser 1995; Lai *et al.*

2008; Schnaufer *et al.* 2002). *T. evansi* and *T. equiperdum* can only survive as bloodstream forms, which produce ATP exclusively through glycolysis (Helfert *et al.* 2001; Roldán *et al.* 2011; Stephens *et al.* 2007).

Based on the restriction enzyme profile on kDNA minicircle, *T. evansi* are grouped into type A (96% sequence identity) and type B that shows >96% identity within the group, and 50–60% identity to type A minicircles (Borst *et al.* 1987; Njiru *et al.* 2006). Isolates with minicircle type A are the most abundant throughout the whole distribution range of *T. evansi* (Bajyana Songa *et al.* 1990; Ou *et al.* 1991; Lun *et al.* 1992). On the other hand, type B minicircles have been detected only in a few rare *T. evansi* isolates from camels from Kenya (Borst *et al.* 1987; Ngaira *et al.* 2005). Some *T. evansi* from South America and China lack both maxicircle and minicircles (akinetoplastic) (Masiga & Gibson 1990; Stevens *et al.* 1989; Ventura *et al.* 2000; Schnaufer *et al.* 2002; Borst *et al.* 1987; Bajyana Songa *et al.* 1990; Ou *et al.* 1991; Lun & Vickerman 1991).

In addition to the natural loss of the kDNA, it is very fragile and highly sensitive to drugs that intercalate into DNA or otherwise interfere with replication giving rise to induced dyskinetoplastic (Dk) strains of trypanosomatids (Schnaufer *et al.* 2002). *T. equiperdum* strains have retained their maxicircles, in some cases with substantial deletions, but have lost their minicircle diversity (Lai *et al.* 2008; Schnaufer *et al.* 2002). In these dyskinetoplastic strains, in addition to its role in ATP production (through oxidative phosphorylation), specific mutations (L262P and A273P) in the nuclearly encoded F_0F_1-ATP synthase gamma (γ) subunit compensate for loss of kDNA-encoded gene products in the bloodstream form (BSF) parasite (Dean *et al.* 2013). *T. evansi* and *T. equiperdum* are morphologically indistinguishable from each other and from the long slender bloodstream from *T. b. brucei*, and their status as independent species has been questioned (Brun *et al.* 1998; Lai *et al.* 2008; Claes *et al.* 2005). Recently, sequencing of the genome of an akinetoplastic *T. evansi* strain from China (STIB 805) in comparison with the *T. b. brucei* reference strain (TREU 927/4), showed extensive similarity and the phylogenetic analysis indicated that *T. evansi/T. equiperdum* evolved from within the *T. brucei* group on at least four independent occasions and from genetically distinct *T. brucei* strains (Carnes *et al.* 2015). Moreover, a phylogenetic analysis based on RNA repeats from various isolates of *T. evansi, T. equiperdum*, *T. b. brucei* and *T. b. gambiense* showed no species-specific clusters (Lai *et al.* 2008). In conclusion, there is strong recommendation for re-classification of *T. evansi* and *T. equiperdum* as *T. brucei* subspecies, i. e. *T. b. evansi* and *T. b. equiperdum* respectively (Carnes *et al.* 2015; Lai *et al.* 2008; Claes *et al.* 2003a; Claes *et al.* 2005).

To understand the genetic heterogeneity of *T. evansi*, considerable studies targeting the analysis of isoenzymes, restriction fragment length polymorphism (RFLP), microsatellite markers and random amplified polymorphic DNA (RAPD) indicated that *T. evansi* isolates from different parts of the globe are genetically homogeneous (Gibson *et al.* 1983; Stevens *et al.* 1989; Bajyana Songa *et al.* 1990; Biteau *et al.* 2000; Lun *et al.* 2004; Ventura *et al.* 2002). *T. evansi* type A is believed to exist as a single clonal lineage (Gibson *et al.* 1983; Njiru *et al.* 2007; Boid 1988). This

low heterogeneity was partly attributed to the use of techniques with low resolution and to the absence of recombination caused by the fact that genetic exchange in trypanosomes only occurs during their development in the tsetse fly which is not the case for *T. evansi* (Jenni *et al.* 1986; Njiru *et al.* 2007). On the other hand, due to extended host pleotropism in diverse geographical regions, heterogeneity in virulence and pathogenesis, significant genetic variability is to be expected (Reid 2002; Queiroz *et al.* 2000; De Menezes *et al.* 2004). Recent studies through AFLP, inter-simple sequence repeats (ISSR), microsatellites and ITS region analysis indicated that *T. evansi* type B is genetically divergent from *T. evansi* type A (Masiga *et al.* 2006; Njiru *et al.* 2007; Amer *et al.* 2011).

4. Variant surface glycoprotein (VSG) and antigenic variation

The VSGs, anchored to the cell surface through a covalent bond between the C-terminal residue and glycosylphosphatidylinositol (GPI) in the cell membrane, with estimated 10^7 molecules per cell, form a 12-15 nm monolayer over the entire surface of the BSF trypanosomes and is an essential virulence factor (Vickerman 1969; Ferguson *et al.* 1988). Each VSG molecule contains an N-terminal and a C-terminal domain (Johnson & Cross 1979; Carrington *et al.* 1991). The N-terminal domain is exposed to the extracellular environment and shows extreme variability in primary sequence of 350-400 residues. The relatively more conserved C-terminal domain consits of approximately 50-100 residues, but is inaccessible to antibodies and thus unlikely affects antigenic variation (Miller *et al.* 1984; Schwede *et al.* 2011). The highly immunogenic VSG determines the variable antigen type (VAT) of the individual trypanosome and elicits VAT specific protective antibodies with opsonizing, agglutinating and lytic activity (Van Meirvenne *et al.* 1995; Schwede *et al.* 2015; Schwede *et al.* 2011). RoTat 1.2 is the predominant VAT of most *T. evansi* strains (Bajyana Songa & Hamers 1988; Verloo *et al.* 2001). To deal with host immune pressure, trypanosomes have evolved a system called antigenic variation (Horn 2014; Morrison *et al.* 2009; Pays *et al.* 2004; Vickerman 1978). Antigenic variation is a periodic switch in the VSG expression, whereby the parasites sequentially express and shed a series of different VSGs, that enables them to evade the host's protective immune responses (Vickerman 1978). A single parasite expresses only one type of VSG at a given time, except during switching (Barry *et al.* 2005). During the first ascending wave of parasitaemia, the majority of the parasites express the same VSG or the major VAT (Hall *et al.* 2013; Robinson *et al.* 1999). Then approximately 1% of trypanosome divisions produce a new VAT by expressing a different VSG (Robinson *et al.* 1999; Hall *et al.* 2013). These new 'antigenically distinct' trypanosomes multiply and replace the first VAT, giving rise to a subsequent parasitaemia wave which is repeated multiple times and results in the development of a chronic infection (Pays *et al.* 2001; Baral 2010; Schwede & Carrington 2010; Hall *et al.* 2013). The waves of parasitemia in the infected hosts are the result of continuous interplay between the immune system and antigenic variation. In addition, VSG switching allows the parasites to infect the host that has antibodies against other

previously infecting variants (Barry *et al.* 2005). Each individual growth peak can contain several distinct variants (Figure 1.4) (Cnops *et al.* 2015).

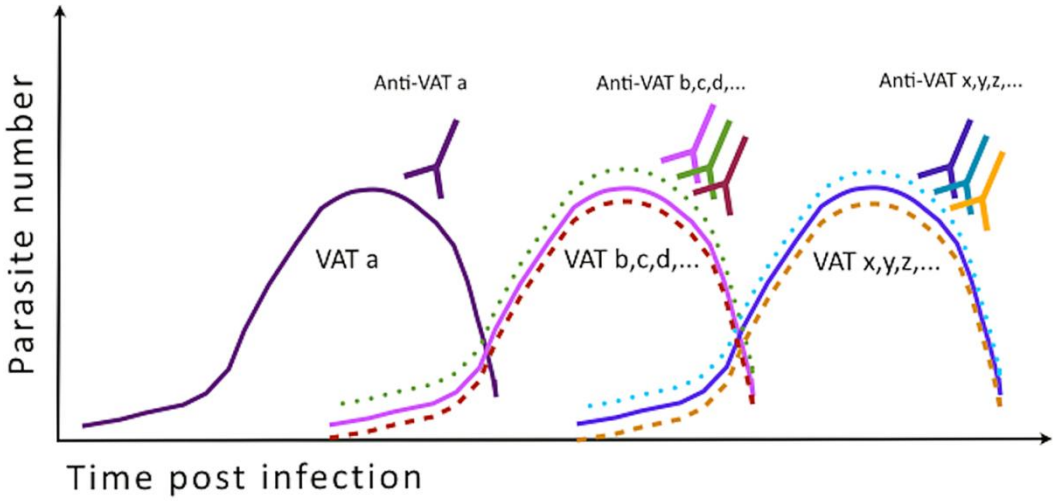

Figure 1.4: Representation of the concept of antigenic variation during mammalian *T. brucei* infection (Cnops *et al.* 2015).

5. Some non-variable surface proteins

5.1. Invariant surface glycoprotein 75 (ISG75)

The VSG dimers act as a protective umbrella for underlying surface molecules such as invariant surface glycoproteins (ISGs). The bloodstream forms of trypanosomes contain about 5 x 10^4 glycosylated ISG75 (ISG75) molecules, with an apparent molecular mass of 75 kDa and distributed over the entire cell surface of *T.b. brucei* (Ziegelbauer & Overath 1992; Ziegelbauer *et al.* 1992; Tran *et al.* 2008; Overath *et al.* 1994). The immature ISG75 polypeptide of 523 amino acid residues is comprised of four main regions: an N-terminal hydrophobic signal sequence (28 amino acids) that is cleaved off yielding a mature protein starting at Glu29; a large hydrophilic extracellular domain; a stretch of 20 hydrophobic residues close to the C-terminus forming a single trans-membrane α-helix; and a small hydrophilic domain (29 amino acids) exposed on the cytoplasmic face of the plasma membrane (Ziegelbauer *et al.* 1995). Multiple copies of *ISG75* are present in the genome and are transcribed in all species and subspecies of *Trypanozoon* with varying copy number among species, ranging from at least 4 to 16 copies per genome. Based on nucleotide similarity, ISG75 is divided into Group I and Group II with 77% and 75% identity respectively (Tran *et al.* 2006).

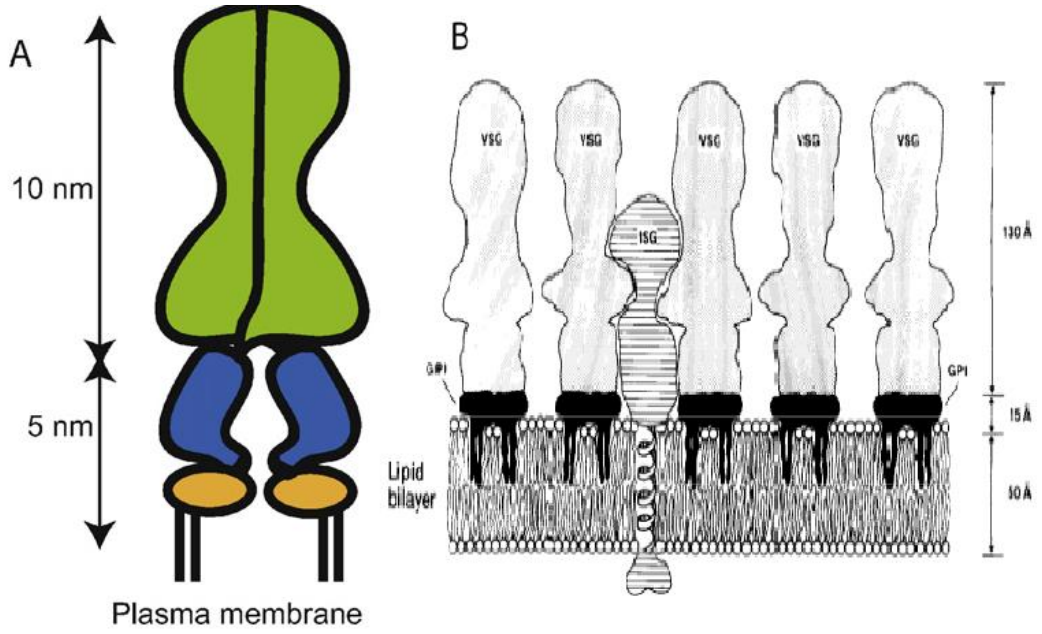

Figure 1.5: A. Schematic representation of a VSG dimer: The N-terminal domain is depicted in green, the C-terminal domain in blue and the GPI-anchor in yellow. B. Organization of dimeric variant surface glycoprotein molecules anchored in the membrane by glycosylphosphatidylinositol (GPI) residues. The hypothetical arrangement of an ISG molecule with a membrane spanning α–helix between the VSG molecules is shown. There is only one ISG for approximately 100 VSG molecules. Adapted from Overath *et al.* (1994) and Schwede *et al.* (2011).

5.2. Invariant surface glycoprotein 65 (ISG65)

ISG65 was identified together with ISG75 in the same experiment by surface biotinylation (Ziegelbauer & Overath 1992; Ziegelbauer *et al.* 1992). ISG65, with apparent molecular mass of 65 kDa, is a BSF specific protein of *T. b. brucei* but its function remains unknown (Ziegelbauer & Overath 1992; Jackson *et al.* 1993). ISG65 is uniformly spread over the entire cell surface, with an estimated 5-7 x 10^4 molecules per cell (Ziegelbauer & Overath 1992; Ziegelbauer *et al.* 1992; Jackson *et al.* 1993). The ISG65 gene codes for a polypeptide of 436 amino acid residues with an N-terminal cleavable signal sequence, a large hydrophilic extracellular domain, and a hydrophobic transmembrane α-helix followed by a small intracellular domain. The gene is present in multiple copies, arranged in tandem repeats (Ziegelbauer *et al.* 1992). ISGs are accessible by immunoglobulins but binding is limited and tolerated by the trypanosome (Schwede *et al.* 2015).

5.3. Cytoskeletal tandem repeat protein GM6

Tandem repeat (TR) proteins of trypanosomatid parasites are often targets of B cell responses (Goto *et al.* 2007). Tandem repeat (TR) protein GM6 is a cytoskeletal protein, located at the connection site between the microtubules of the membrane skeleton and the flagellum of the parasite (Figure 1.6).

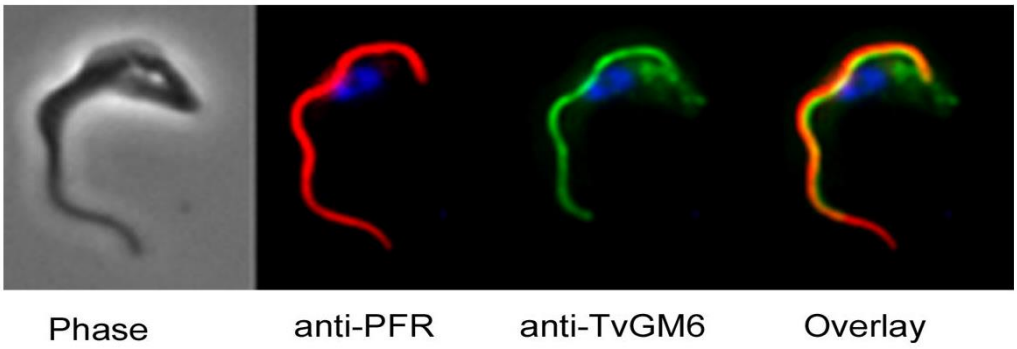

Figure 1.6: Phase contrast and immunofluorescence pictures of a *T. vivax* trypanosome showing partial co-localisation of GM6 and the paraflagellar rod (PFR) proteins by means of specific fluorescent antibodies (Pillay *et al.* 2013).

GM6 is equally present in bloodstream and procyclic forms of trypanosomes, and is well conserved between different species of salivarian trypanosomes and, though somewhat less, in the stercorarians *T. rangeli* and *T. cruzi* (Müller *et al.* 1992; Pillay *et al.* 2013). GM6 which exerts structural roles in the trypanosomal cell consists of repetitive sequence motifs of 60, 11, 9 amino acids in *T.b. brucei, T. vivax* and *T. congolense* respectively (Pillay *et al.* 2013; Müller *et al.* 1992). *T. congolense* GM6 shares 63.8% identity with *T.b. brucei* GM6 while the *T. vivax* GM6 repeat sequence shares only 51 and 55% identity and 72 and 64% similarity with the homologs of *T. b. brucei* and *T. congolense*, respectively (Nguyen *et al.* 2012; Pillay *et al.* 2013; Nguyen *et al.* 2014).

GM6 is recognized by B-cells when parasites are destroyed by the host immune response (Müller *et al.* 1992; Imboden *et al.* 1995). However, it has been observed that the antibody response against GM6 decreases to baseline approximately one month after treatment. In the absence of antigenic stimulation, when the parasitaemia drops beneath the necessary parasite load, the antibody response is short-lived (Pillay *et al.* 2013).

5.4. Drug transporters

Trypanosomes have two high-affinity adenosine transporters: a P1 type, which transports inosine and accounts for 60–70% of the total adenosine uptake; and a P2 type, which transports adenine and accounts for 30–40% of the total adenosine uptake into the cell. Diamidines are

transported via the P2 transporter (Anene *et al.* 2001). The *T. evansi* adenosine transporter-1 gene (*TevAT1*) (which shares 99.7% homology with TbAT1 gene in *T. brucei*) encodes a P2-like nucleoside transporter required for the uptake and/or action of berenil in *T. evansi.* TbAT1 is also involved in melarsoprol uptake (Burkard *et al.* 2011). On the other hand, the high-affinity pentamidine transporter 1 (HAPT1), today recognized as aquaglyceroporin 2 (aqp2) is responsible for most of the P2-independent diminazene uptake in bloodstream trypanosomes and its absence generally correlates with high levels of diamidine resistance (Teka *et al.* 2011; Baker *et al.* 2013). Melaminophenyl arsenicals such as cymelarsan are transported into the trypanosome by the P2 adenosine/adenine transporter and additionally by the aquaglyceroporins (aqp2/3) (De Koning 2008; Alsford *et al.* 2012; Carter & Fairlamb 1993). Mutations in aquaglyceroporin 2 correlate with decreased susceptibility to pentamidine and melarsoprol (Graf *et al.* 2013). The ISG75, acts as a major receptor for suramin (or the serum component to which it is bound) delivering the drug into the degradative arm of the endocytic pathway (Alsford *et al.* 2012; Alsford *et al.* 2013). No transporters are known to exist for isometamidium chloride (ISM). ISM freely crosses the plasma membrane, probably by facilitated diffusion, and is subsequently actively accumulated into the mitochondria, using the mitochondrial potential as a driving force. Resistance to ISM is mostly associated with cross-resistance to homidium (De Koning 2001; Peregrine *et al.* 1997). Recently, innate resistance of *T. evansi* to ISM has been observed to relate with the A281 deletion in the ATP F1 γ subunit gene (Gould & Schnaufer 2014). Moreover, RNA silencing in *T.b. brucei* revealed that depletion of vacuolar ATPase or adaptin-3 subunits is associated with ISM resistance (Baker *et al.* 2015).

6. Interactions between the trypanosome and the mammalian host

In contrast to trypanosomosis due to *T. brucei*, information on the immunobiological aspects and parasite control mechanisms of *T. evansi* infection is limited (Onah *et al.* 1998b; Onah *et al.* 1998a). Unlike to cyclically transmitted trypanosomes, the mechanically transmitted *T. evansi* parasites complete their entire life cycle in the mammalian host and are under constant immune pressure (Baral *et al.* 2007). Co-evolution has resulted in the development of well-balanced growth regulation systems, allowing the parasite to survive sufficiently long without killing its mammalian host, ensuring its efficient transmission (Stijlemans *et al.* 2010). Upon infection with African trypanosomes, both arms of the host immune system are activated comprising (i) a strong type I cellular immune response, consisting of pro-inflammatory molecules such as tumor necrosis factor (TNF), interleukines (IL-1, IL-6) and nitirc oxide (NO) produced mainly by "classically" activated macrophages and (ii) a strong humoral anti-trypanosome B-cell response (Mansfield & Paulnock 2005; Magez *et al.* 2008).

Similar to *T. brucei* and *T. congolense* infection, *T. evansi* infection induces immunosuppression at the level of antibody production against heterologous antigens and of the proliferative response of peripheral blood lymphocytes (Holland *et al.* 2001; Holland *et al.* 2003; Onah *et al.* 1998b; Onah *et al.* 1996; Onah *et al.* 1999).

IFN-γ dependent NO production is involved in the suppression of T cell proliferation in *T. evansi* and *T. brucei* infection (Hertz & Mansfield 1999; Beschin *et al.* 1998). However, this suppression had no measurable effect on parasitemia control or on the life span of *T. evansi* infected mice under laboratory conditions (Baral *et al.* 2007). The dramatic suppression of the immune responses might result in a high susceptibility to opportunistic infections (Darji *et al.* 1992; Flynn & Sileghem 1991; Sileghem *et al.* 1991). Moreover, immunosuppression due to *T. evansi* was shown to cause vaccination failure against classical swine fever and *Pasteurella multocida* (haemorrhagic septicemia) (Holland *et al.* 2003; Holland *et al.* 2001). The mechanism of immunosuppression in trypanosome infected animal/human is reviewed well by Baral (2010). Both macrophages and T cells are involved in initiation of immunosuppression (Tabel *et al.* 2008). The immunosuppression caused by suppressive macrophages is characterized by an inhibition of the T cell proliferation due to down regulation of both IL-2 production and expression of IL-2 receptor (Sileghem *et al.* 1989; Darji *et al.* 1992).

Trypanotolerance is the relative capacity of some livestock breeds to survive, reproduce and remain productive under trypanosomosis challenge without the aid of trypanocidal drugs. Trypanotolerant cattle such as the N'Dama, the short-horn taurine Baoulé and Lagune, control the development of the parasites and limit their pathological effects, the most prominent of which is anaemia (D'leteren *et al.* 1998; Murray & Dexter 1988). Trypanotolerance is under genetic control, but its stability can be affected by environmental factors, such as overwork, intercurrent disease and repeated bleeding, pregnancy, parturition, suckling, lactation and malnutrition (Berthier *et al.* 2015). The capacity of trypanotolerant cattle to generate sustained antibody responses to trypanosome antigens is probably the most prominent immunological feature that has been identified so far. Following infection, animals develop a trypanosome-specific IgM response that is similar in both trypanotolerant and trypanosusceptible cattle (Authié *et al.* 1993; Williams *et al.* 1996). A distinct population of IgM consists of antibodies of low specificity, which react with both trypanosome and non-trypanosome antigens. These polyspecific antibodies, which may contain auto-antibodies are likely to mediate pathology rather than protection (D'leteren *et al.* 1998; Williams *et al.* 1996). A trypanosome-specific IgG response (predominantly IgG1) is elicited in infected cattle almost coincidentally with the IgM response. Besides having a greater ability to develop specific humoral responses, trypanotolerant cattle have been found to maintain higher complement levels during trypanosome infection than susceptible zebu cattle (Authié & Pobel 1990). The bone marrow of trypanotolerant breeds has higher intrinsic capacity to respond to anaemia (Andrianarivo *et al.* 1995; Andrianarivo *et al.* 1996).

7. Epidemiology and economic importance of *T. evansi* infection

The epidemiology, pathogenesis and economic significance of surra, due to *T. evansi* infection is descibed well in recent reviews (Figure 1.7) (Desquesnes *et al.* 2013b; Desquesnes *et al.* 2013a). Surra is widely distributed in Afica, Middle East, Latin America, and Asia with sporadic import cases in Europe (Hoare 1972; Gutiérrez *et al.* 2010). Surra is one of the OIE list B multiple species diseases (OIE 2016). This multi-host characteristic is attributed to the fact that the mechanical vectors such as tabanids do not have strict host preference (Muzari *et al.* 2010).

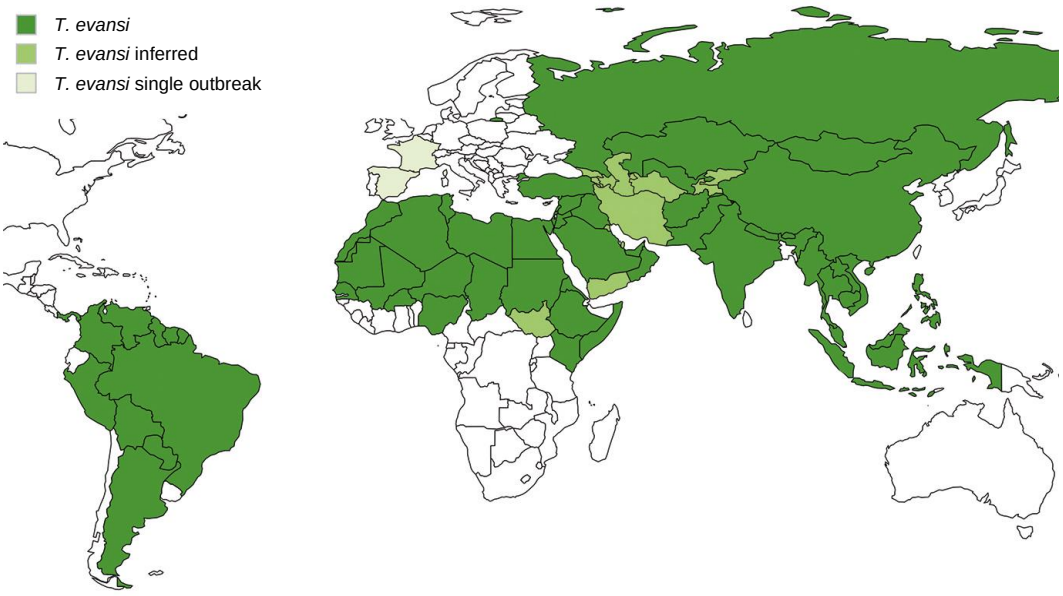

Figure 1.7: Geographical distribution of Trypanosoma evansi in the world (Auty *et al*. 2015).

In the non tsetse belt of Africa, surra is principally a disease of camels and horses but cattle and goats are also highly susceptible (Gutiérrez *et al.* 2006b). There is seasonal influence on epidemics related to seasonal activity of vectors and other factors such as stress from overwork, food shortages, and/or insufficient or poor quality water (Dia *et al.* 1997a; Desquesnes *et al.* 2013a; Zeleke & Bekele 2001). The distribution of *T. evansi* infection in Ethiopia follows the distribution of dromedary camels (Figure 1.8) (Dagnatchew 1982; Abebe 2005). However, due to logistic deficiency and lack of accurate diagnostics for the disease, the exact burden and economic importance of the disease is not well known. Recent studies in pocket areas of Ethiopia indicated parasitological (2%, 12 %) and serological (24%, 25%) prevalence in camels respectively from Oromia and Afar regions (Fikru *et al.* 2015; Hagos *et al.* 2009).

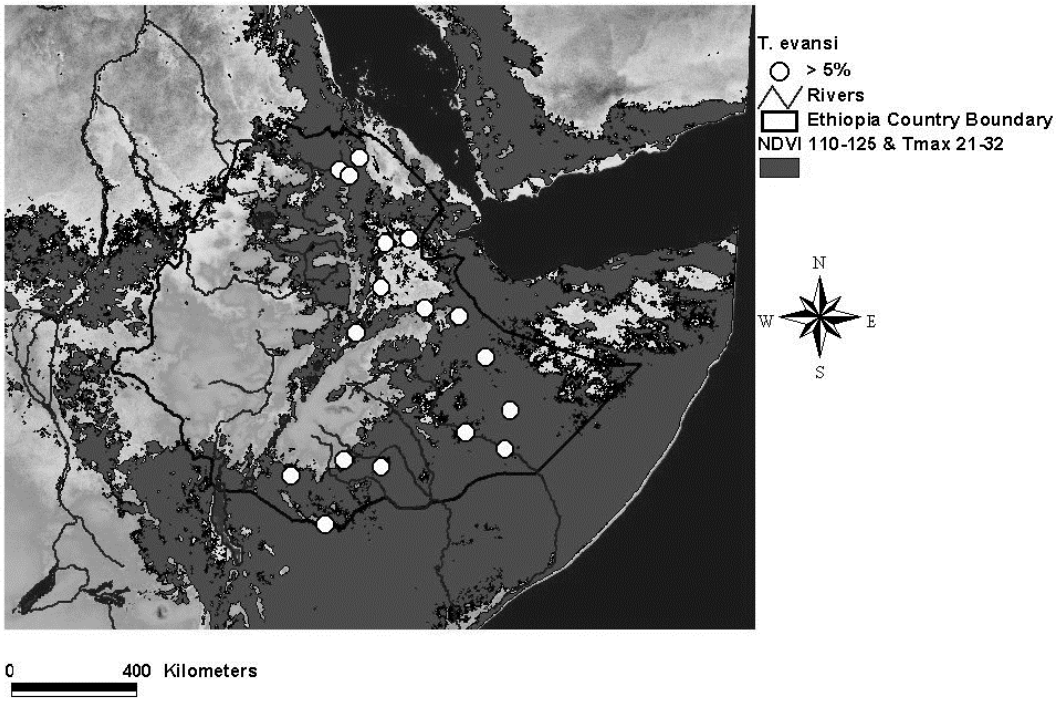

Figure 1.8: Geographical distribution of *Trypanosoma evansi* in Ethiopia (white circular dots) (Abebe 2005).

In the Middle East and towards Asia, the geographical distribution of *T. evansi* is also closely related to that of dromedaries. Surra is widely distributed principally in bovines, camels, buffaloes and equines in large areas of India (Hoare 1972; Ravindran *et al.* 2008; Singh *et al.* 2004; Pathak *et al.* 1993; Sumbria *et al.* 2014; Sharma *et al.* 2013; Kundu *et al.* 2013; Ul Hassan *et al.* 2006; Shahzad *et al.* 2010; Tehseen *et al.* 2015).

In Latin America, *T. evansi* is principally a disease of horses and bovine and induces outbreaks with very high morbidity and mortality. Other domestic species that are affected by surra are buffaloes, cats, pig and dogs (Aquino *et al.* 2010; John *et al.* 1992; Aref *et al.* 2013; Defontis *et al.* 2012; Rjeibi *et al.* 2015; Stevens *et al.* 1989; Raina *et al.* 1985). The wild reservoirs in Latin America are wild pigs (*Tayassu tajacu*), white tail deer (*Odocoileus virginianus chiriquensis*), coati (*Nasua nasua*), brocket deer (*Mazama satorii*), vampire bats (*Desmodus rotundus*), capybaras (*Hydrochoerus hydrochaeris*), guinea pig (*Cavia porcellus*), wild dog (*Canis azarae*), ocelot (*Felis pardalis*) and llamas (Desquesnes *et al.* 2013a).

In Asia, the geographical distribution of *T. evansi* is spreading steadily in large areas in India, China, and Russia (Lun *et al.* 1993; Singh *et al.* 2004). Surra usually exhibits an endemic and chronic nature, however, an acute outbreaks can occur when the disease is introduced into new

animal population with no prior exposure (Berlin *et al.* 2010; Gutiérrez *et al.* 2005; Adrian *et al.* 2010; Desquesnes *et al.* 2008).

T. evansi is not present in Australia, but it may spread eastward from Indonesia to Papua New Guinea and then Australia (Reid & Copeman 2000). Surra cases in Europe have been ascribed to importation of camels from the Canary Islands where the disease was first diagnosed in 1997, in a dromedary camel imported from Mauritania (Gutiérrez *et al.* 2000). Many camels had been imported from the Canaries to the European mainland without any previous examination to detect *T. evansi* infection (Gutiérrez *et al.* 2000; Gutiérrez *et al.* 2010). This has caused two outbreaks of *T. evansi* infection, in metropolitan France in 2006 on a sheep and camel farm and in Spain in 2008 (Desquesnes *et al.* 2008; Tamarit *et al.* 2010).

T. evansi cannot infect human because of its susceptibility to the trypanolytic factor (TLF) in normal human serum (NHS), apolipoprotein L-1 (ApoL-1) that provides innate protection of humans from infection by African trypanosomes, such as *T. evansi*, *T. b. brucei,* and others, with the exception of *T. b. rhodesiense* and *T. b. gambiense,* which developed resistance mechanisms (Vanhollebeke *et al.* 2008; Pays *et al.* 2006; Vanhamme *et al.* 2003). In India, a human case of trypanosomosis due to *T. evansi* occurred in a person with frameshift mutations in both Apo L-1 alleles that led to an unexpected termination of protein translation by internal stop codons which resulted in a total absence of Apo L-1 (World Health Organization (WHO) 2005; Joshi *et al.* 2005; Powar *et al.* 2006; Vanhollebeke *et al.* 2006). More recently, a woman in Vietnam, with apparently normal blood concentrations of functional Apo L-1 was diagnosed with *T. evansi* infection suggesting that other host parameters may play a role in susceptibility to *T. evansi* infection (Van Vinh *et al.* 2016).

T. evansi is mechanically transmitted by blood sucking insects and requires high parasitaemia of the “donor host” (Desquesnes *et al.* 2013a). Of all, mechanical transmission by biting insects such as tabanids and *Stomoxys* is the most important mode of transmission. Besides vector transmission and the contamination of a wound, iatrogenic transmission caused by the use of nonsterile surgical instruments or needles may be of importance, especially during vaccination campaigns and mass treatments. Per-oral transmission through eating infected prey was reported in tigers, dogs and rodents (Moloo *et al.* 1973; Raina *et al.* 1985; Desquesnes *et al.* 2013a). In Latin America, vampire bats (*Desmodus rotundus)* can act as vector of *T. evansi*. They are infected orally when taking blood from an infected prey. As a host of *T. evansi*, bats may develop clinical symptoms and die during the initial phase of the disease. However, in bats that survive, parasites multiply in the blood and are found in the saliva from where they can be transmitted to another host during biting (Hoare 1972; Desquesnes 2004). Recently, vertical transmission of *T. evansi* in naturally infected camels and in experimentally in sheep has been documented (Narnaware *et al.* 2016; Campigotto *et al.* 2015). Clinical signs across host species are detailed below in section 9.1.

The cumulative effects of the different pathologies due to *T. evansi* infection cause serous economic losses due to its impact high mortality, reduced production (milk and meat), reduced reproductive performance, poor carcass quality, decreased draught power and immunosuppression in livestock. Furthermore, the financial expenditures for use of chemotherapeutic interventions and replacement stocks is quite high (Reid 2002; Pholpark *et al.* 1999; Payne *et al.* 1991; Salah *et al.* 2015).

8. Control of African Animal Trypanosomosis

8.1. Trypanocidal treatment

Contol of vector borne diseases targets both disease control and vector control. As no vaccine against *T. evansi* infection exists, disease control is mainly based on trypanocidal drugs. Each year, 35 million doses of veterinary trypanocidal drugs are administered in Africa, with isometamidium chloride (ISM), diminazene aceturate (DA) and ethidium bromide (EtBr) representing respectively 40%, 33% and 26% of the total trypanocidal drug market by value (Geerts & Holmes 1998). “Curative trypanocidals” have a short term effect, while “chemoprophylactic trypanocidals” not only kill parasites but also protect against infection due to a sustained curative drug level in the serum of treated animals (Table 1.1) (Desquesnes *et al.* 2013a). DA is affordable and easily accessible which often makes it the first-line treatment. DA can be used as “premunition treatment” at which the host is clinically cured but remains infected, however this could contribute for selection of drug resistant strains. This treatment regime could be used in highly enzootic situations, when the infection is not lethal, such as *T. evansi* in bovines while “sterilizing” treatment is used for lethal *T. evansi* infection in horses and dogs (Desquesnes *et al.* 2013a). ISM, synthesized by coupling homidium with a part of the diminazene molecule, has been used in the field for several decades prophylactically or therapeutically (Leach & Roberts 1981). ISM is mainly accumulated in the kinetoplast, whereas homidium is spread much more diffuse throughout the trypanosome (Boibessot *et al.* 2002). EtBr (or chloride) is a highly toxic, DNA intercalating agent and has mutagenic action (Macgregor & Johnson 1977). The mode of action of DA is not clear while for ISM, it cleaves kDNA-topoisomerase complexes, causing the desegregation of the minicircle network within the kinetoplast. However, Kaminsky *et al*. showed that dyskinetoplastic trypanosomes are equally sensitive to ISM as kinetoplastic trypanosomes thus questioning the relevance of the mode of action of ISM on the kDNA (Shapiro & Englund 1990; Girgis-Takla & James 1974; Kaminsky *et al.* 1997).

Suramin appeared in 1920 as drug against the early stage of sleeping sickness (HAT) and does not cross the blood-brain-barrier (Nok 2003; Sanderson *et al.* 2007). In addition to the many other intracellular effects that suramin may exert on the parasite, it exerts inhibitory activities on a wide spectrum of enzymes, e.g. inhibition of the uptake of low density lipoproteins (LDL)

(Vansterkenburg *et al.* 1993; Wang 1995). Suramin has been used to treat surra in Sudan and Kenya (El Rayah *et al.* 1999; Otsyula *et al.* 1992).

Quinapyramine was introduced in the 1950s and is used as a therapeutic (antrycide sulphate) and prophylactic drug (antrycide prosalt) for *T. evansi* in camels and *T. evansi* and *T. equiperdum* in horses. However, due to development of drug resistance, it was withdrawn from the market in Africa in the 1970s. The drug has been re-introduced on the market in the mid 1980s under two different names. One of the products, tribexin prosalt (quinapyramine sulphate:quinapyramine chloride, in the ratio of 3:4; Indian Drugs and Pharmaceuticals Ltd, Hyderabad, India) is recommended to treat *T. evansi* infections in donkeys and camels. Another product, trypacide (May and Baker, UK), is available in two forms, trypacide sulphate (subcutaneous, curative) and trypacide pro-salt (quinapyramine sulphate:quinapyramine chloride, in the ratio of 3:2, prophylactic) (Kinabo 1993).

Melarsamine hydrochloride (MelCy) is a water-soluble trivalent arsenical agent patented in 1985 under the trade name cymelarsan (Rhone Merieux, France) (Berger & Fairlamb 1994; Otsyula *et al.* 1992).

Table 1.1: Trypanocidal drugs used for treatment of surra in various host species (Desquesnes *et al.* 2013a; De Koning 2001; Delespaux & De Koning 2007; Kinabo 1993; Röttcher *et al.* 1987).

Trypanocidal drug	**Trade name**	**Family**	**Therapeutic/Prophylactic/route and dosage**	**Host species**
Diminazene (di)aceturate (DA)	Veriben, Berenil	aromatic diamidines	therapeutic (7mg/kg bw, IM), prophylactic (3.5 mg/kg bw)	ruminants, poor efficacy and tolerance in horses and dogs
Isometamidium (ISM) chloride (hydrochloride)	Samorin, Trypamidium, Veridium	phenanthridines	therapeutic (0.5mg/kg bw, IM), prophylactic (1mg/kg bw)	ruminants, horses have a limited tolerance
homidium chloride (bromide)	Novidium (Ethidium)	phenanthridines	prophylactic (1 mg/kg bw, IM)	ruminants, horses have a limited tolerance
Quinapyramine dimethyl-sulphate	Antrycide sulphate	aminoquinaldines	therapeutic (8 mg/kg bw, SC), prophylactic (5mg/kg bw)	horses and camels
Suramin	Naganol	ureic, sulfonated naphthylamine	therapeutic (7-10 mg/kg bw, IV in horses, and 8-12 mg/kg bw, IV in camels), prophylactic (1-2 g repeated at 10 days intervals)	horses and camels
Melarsomine dihydrochloride	Cymelarsan	arsenical	therapeutic 0.25, 0.25–0.5, 0.5, 0.75, and 2.5 mg/kg bw, IM respectively for each host species	camels, horses, cattle; buffaloes, dogs

bw: body weight, IM: intramuscular, IV: intravenous, SC: subcutaneous

Two injections of cymelarsan (0.5 mg/kg bw), with an interval of 1 month, is recommended to control *T. evansi* outbreaks in non-endemic areas (Gutiérrez *et al.* 2014). Alternate use of DA and ISM constitutes a "sanative pair," which means that once resistance develops to one of the drugs, the other drug is still effective to control the infection. Most trypanocidal drugs cannot effectively cure animals with advanced disease, particularly with nervous involvement, when the parasite has infiltrated extravascular spaces. This could be due to rapid clearance from the circulation or inability to cross the blood-brain-barrier (Desquesnes *et al.* 2013a).

Control of AAT is hampered by emergence of drug-resistant trypanosomes (Mäser *et al.* 2003; Geerts *et al.* 2001). Extensive use of DA, ISM, quinapyramine and suramin has resulted in the appearance of resistant strains (Leach & Roberts 1981; Kinabo 1993; Röttcher *et al.* 1987). Drug resistance can be innate (resistant individuals without previous exposure to the particular drug), or acquired (due to drug exposure/pressure, cross-resistance or sometimes by mutagenesis) (Frommel & Balber 1987; Osman *et al.* 1992). Resistance to DA and ISM is reported in various *Trypanosoma* species from different parts of Africa, including in Ethiopia (Moti *et al.* 2012; Dagnachew *et al.* 2015b; Moti *et al.* 2015; Miruk *et al.* 2008). Resistance to ISM is mostly associated with cross-resistance to homidium, as they are structurally related compounds that share the same uptake mechanism (Peregrine *et al.* 1997). Resistance of *T. evansi* to suramin has been observed experimentally in Sudan and China, and camels in Kenya (El Rayah *et al.* 1999; Zhou *et al.* 2004; Otsyula *et al.* 1992). No cross-resistance of suramin with arsenicals, diamidines, quinapyramine or isometamidium was observed (El Rayah *et al.* 1999; Zhou *et al.* 2004; Ross & Barns 1996). So far there are no reports about development of resistance against cymelarsan. This drug is not registered in Ethiopia but it has been used to treat horses infected with *T. equiperdum* (Hagos *et al.* 2010c). However, in the case of nervous infections, high doses (0.5 mg/kg bw), in horses and dogs (2 mg/kg bw), failed to cure the animals (Desquesnes *et al.* 2011; Berlin *et al.* 2010; Desquesnes *et al.* 2013a). Lower doses of cymelarsan failed to treat surra cases in buffaloes (0.25 mg/kg to 3 mg/kg), goats (0.3 mg/kg), mice (0.25 mg and 0.5 mg/kg) and cattle (0.5 mg/kg) (Lun *et al.* 1991; Payne *et al.* 1994a; Hagos *et al.* 2010c; Zweygarth *et al.* 1992; Syakalima *et al.* 1995). Clones of *T. evansi* that developed resistance to cymelarsan in mice showed cross resistance to diminazene and pentamidine (Osman *et al.* 1992; Zweygarth *et al.* 1990).

8.2. Vector control

In addition to treatment, prevention of infection through vector control is possible. The control of mechanical vectors is difficult because of their diversity, high mobility and prolificacy (Foil & Hogsette 1994). Control of tabanids is rarely attempted, because it is costly, unsatisfactory, unsustainable, and does not provide 100% protection against infection (Desquesnes *et al.* 2013a). Nzi and Vavoua traps are used to study insects and monitor control campaigns. The Nzi trap can catch large tabanid species and *Stomoxys*, while the Vavoua trap catches small tabanid species, such as *Chrysops* (deer flies) and *Stomoxys* (Mihok 2002).

8.3. Vaccination

Antigenic variation of trypanosomes is a major obstacle in the development of efficient vaccines (Magez & Radwanska 2009). Initial vaccination experiments on metacyclic variable antigen types (M-VATs) of *T.b. brucei* and VSG of *T. congolense* that were reported to provide some VSG-specific protection were unsuccessful (Crowe *et al.* 1984; Crowe *et al.* 1983; Nantulya *et al.* 1980; Masake *et al.* 1983). Intracellular trypanosome components, such as β-tubulin, actin, microtubule-associated proteins (MAP), purified flagellar pocket fractions, congopain were used for vaccination but none of them was able to induce full protection against infection (Balaban *et al.* 1995; Li *et al.* 2009; Li *et al.* 2007b; Lubega *et al.* 2002; Gull 2003; Mkunza *et al.* 1995; Radwanska *et al.* 2000). Recombinant protein ISG75 has been tried to immunize mice, resulting in high titers of ISG75-specific antibodies. However, challenge with trypanosomes showed no protection and vaccine-induced anti-ISG75 antibody titers decreased rapidly (Magez & Radwanska 2009; Tran *et al.* 2008). All these failures indicate that vaccination against African trypanosomoses might never be feasible (Magez & Radwanska 2009). In addition, all attempts to develop an anti-disease vaccine that protects an infected hosts against the development of severe disease have eventually failed (Hanotte *et al.* 2003; Authié *et al.* 2001; Stijlemans *et al.* 2007; Lalmanach *et al.* 2002; Radwanska *et al.* 2008).

9. Diagnosis of *T. evansi* infection

Knowing the presence or absence of ongoing infection or exposure is important for providing therapy and assessing fit in livestock trade requirements of individual animals and for understanding the epidemiology of a disease within a population. Individual diagnosis, particularly in developing countries, often depends on non-specific clinical signs and/or cumbersome parasitological techniques. It is likely that the diagnosis for individual animals is often missed as clinical symptoms are not pathognomonic and the diagnostic sensitivity of traditional parasitological techniques is low, particularly in the chronic phase of the disease (Büscher 2014; Thrusfield 2007; Jacobson 1998). Each diagnostic test has a number of characteristics. The sensitivity is the proportion of infected animals testing positive -or- sensitivity =true positives/(true positives + false negatives). The specificity is the proportion of non-infected animals testing negative -or- specificity = true negatives/(true negatives + false positives). The reproducibility is the ability of a test to produce consistent results when preformed in different laboratories and the repeatability is the ability of a test to produce consistent results when the test is run on several occasions under identical conditions. Moreover, ease of interpretation, user friendliness, rapidity of results and cost are important parameters (Mabey *et al.* 2004; Jacobson 1998; Thrusfield 2007). Apart from their intrinsic characteristics, the performance of diagnostic tests can be described in terms of positive and negative predictive value (PPV and NPV) that take into account the prevalence of the infection or the disease within a given population. The formulas are as follows:

PPV = sensitivity∗prevalence/[sensitivity∗prevalence +(1-specificity)∗(1-prevalence)] and

NPV= specificity∗(1-prevalence)/[specificity∗(1-prevalence) + (1-sensitivity)∗prevalence].

Hence, the prevalence of an infection will define which type of diagnostic test will perform best in a given population. In areas with high prevalence of a disease, low sensitive and highly specific diagnostics may be adequate if chemoprophylaxis or chemotherapy is administered on a herd basis. In areas with low disease prevalence, more sensitive diagnostic tests are required (Thrusfield 2007). Diagnostic tests for trypanosomes can be generally grouped into 1° direct tests that detect either whole circulating parasites, or, as surrogate of the parasite, its antigens, its DNA or RNA and 2° indirect tests that detect the antibody response of the host to the infection (Büscher 2014).

9.1. Clinical signs and symptoms

The diverse pathologies due to *T. evansi* infection have been well reviewed in (Desquesnes *et al.* 2013b; Habila *et al.* 2012). Briefly, the course of the disease runs from subclinical to acute fatal cases. Severity is related to the difference in virulence of individual strains, susceptibility of the host, local epizootiological conditions and stress factors (Hoare 1972; Desquesnes *et al.* 2013b). In susceptible host species; not the parasite burden itself but immunopathology, including anemia and liver pathogenicity cause mortality and morbidity (Magez *et al.* 2004; Shi *et al.* 2003; Bosschaerts *et al.* 2009; Stijlemans *et al.* 2008). Clinical signs and symptoms in African trypanosomosis are non-specific or may even be obscure, particularly in trypanotolerant breeds. No single symptom is pathognomonic and AAT may simulate many other infections. Therefore, clinical diagnosis remains only presumptive and relies upon the combination of several clinical signs occurring in a susceptible host in a given epidemiological situation (Büscher 2014). At the site where trypanosomes are inoculated by the biting flies, trypanosome multiplication can induce swelling of the skin (chancre) followed by intermittent fever (Luckins *et al.* 1991). Anemia, mainly due to extravascular haemolysis is a cardinal sign of AAT (Stijlemans *et al.* 2008; Rickman & Cox 1983). During late infection, AAT is characterized by loss of condition, lassitude, edema of the lower parts of the body, urticarial plagues and petechial hemorrhages of the serous membranes (OIE 2012; Desquesnes *et al.* 2013b). Abortions, immunosuppression and infiltration and dissemination of *T. evansi* in the central nervous system (CNS) with fatal clinical symptoms are documented (Saleh *et al.* 2009; Holland *et al.* 2003; Holland *et al.* 2001; Seiler *et al.* 1981; Berlin *et al.* 2009).

In camels, surra may be acute with high fever (41°C), weakness, loss of appetite and weight, abortion, oedema (ventral parts, udder or scrotum, and sheath), anaemia with pale mucous membrane, and petechial or ecchymotic haemorrhages and death. It is more often chronic (frequently last 2-3 years) than in horses. Sometimes nervous signs such as periodic convulsion are observed (Gutiérrez et al. 2006a; Gutiérrez et al. 2005). In horses, the incubation period is 1–8 weeks, after which fluctuating fever (41.5°C to 44°C) coincides with high peaks of parasitaemia.

Other symptoms are weakness, lethargy, anaemia, severe weight loss (often accompanied by jaundice and highly coloured urine), transient local or general cutaneous eruption, petechial haemorrhages vulvar and vaginal mucosa and on the anterior chamber of the eye and eyelids, abortion, staggered locomotion and oedema (submaxillary, legs, briskets, abdomen, testicle and sheath or udder) (Silva et al. 1995). Acute signs are often seen in naive populations with high mortality rates above 50%. In enzootic areas, horses may exhibit a certain level of resistance with chronic or subclinical cases and apparently healthy carriers. Donkeys and mules exhibit the same symptoms but milder than those in horses (Silva et al. 1995).

Surra has been considered as a mild, chronic, or asymptomatic disease in cattle and buffaloes, especially in Africa, Venezuela and Latin America, where it is sometimes even difficult to infect animals experimentally (García *et al.* 2006). In India very high mortality rates (>90%) were reported. Common clinical signs include fever, anaemia, losses in weight, milk and meat production, and losses in draught power, abortion, interruption in oestrous cyclicity. Occasionally the evolution may be acute, quickly leading to death (Payne *et al.* 1993; Desquesnes *et al.* 2013b).

In sheep, natural infection is generally considered as mild or asymptomatic. In some cases, experimental infections can even fail, but in others they can lead to clinical signs, mainly fever (40°C), lack of appetite, and anaemia. Parasitaemia is generally low (105 parasites/ml) and decreases until undetectable for several months; however, under certain circumstances such as food restriction or transport stress, parasites can relapse into the blood and clinical signs reappear (Desquesnes *et al.* 2013b). Trypanosomosis including surra in goats may produce acute, subacute, chronic, or subclinical forms. Under natural conditions, goats show mild clinical signs (Gutiérrez *et al.* 2006b). In experimental infection, erratic parasitemia, weight loss, and significant drop in PCV were observed (Ngeranwa *et al.* 1991; Ngeranwa *et al.* 1993).

Infection in pigs has long been reported as very mild or symptomless (including under experimental conditions), however, symptoms such as fever (39°C–41°C), anaemia, weight loss, anorexia, low fertility, emaciation, abortion and cutaneous rash, and late nervous evolution, with hind leg paralysis can be seen.

Dogs are highly susceptible for surra, often exhibiting acute cases with strong clinical signs leading to death (within a week to a month), especially in stray dogs which are not treated and sometimes even despite treatments (Herrera *et al.* 2004; Singh *et al.* 1993). Clinical signs are intermittent fever (39°C–41°C), oedema (head including larynx, abdominal wall and legs), anaemia, weakness, lack of appetite leading to emaciation and, sometimes, paresis of the hindquarters and myocarditis, sexual excitement can be seen. Ocular signs with conjunctivitis, lachrymation, keratitis, corneal opacity, and/or haemorrhagic signs, fibrin deposits in the anterior chamber of the eye; and parasites in ocular aqueous fluid are common (Savani *et al.* 2005; Aref *et al.* 2013).

Confirmed cases of *T. evansi* infection are documented in tigers. Clinically sick tigers showed anorexia, constipation, lethargy and convulsion, rapid respiration, panting, head pressing, rapid pulse, fever (Upadhye & Dhoot 2000). An outbreak of *T. evansi* infection in four tigers, two jaguars, and one leopard was characterized by sudden death, respiratory distress, running nose and convulsions (Sinha et al. 1971). Very little is known about natural infection in cats, but *T. evansi* experimental infection in cats induced only mild symptoms, such as fever, apathy, hyporexia, and vomiting as well as muscular pain, hyperproteinaemia, hyperglobulinaemia, and hypoalbuminaemia (Da Silva et al. 2010b; Da Silva et al. 2009).

9.2. Parasitological techniques

Parasitological tests are in principle 100% specific and therefore can be used for confirmation diagnosis. On the basis of morphological characteristics, microscopy allows tentative identification of the trypanosome species. *Trypanozoon* parasites, including *T. evansi* are found intravascularly as well as extravascularly in different tissues, including the central nervous system. Trypanosomes that circulate in the blood are the most accessible for parasitological examinations. In the chronic phase of an infection, the parasite load in the blood often remains below the detection limit of common microscopic techniques thus making parasite detection cumbersome and poorly sensitive (Büscher 2014).

The wet blood film and stained thin and thick smears are direct blood examination techniques without any concentration step. The major disadvantage of these tests is the low sensitivity as only parasite levels above 100,000/ml can be detected (Paris *et al.* 1982). Preparation and microscopic examination of the stained slides is time-consuming (10 to 20 min per slide) and requires a certain level of expertise to recognize the parasite (Chappuis *et al.* 2005). The micro haematocrit centrifugation technique (mHCT) or Woo test allows the concentration of trypanosomes in the blood and therefore is a more sensitive parasite detection method. However, identification of the trypanosome species is not obvious although the morphology and particularly the characteristic movement of the parasites may be indicative. In this technique, blood is drawn into anticoagulant-coated capillary tubes and centrifuged at high speed about 13,000 g) for 5 minutes to concentrate the trypanosomes in the buffy coat layer (Woo 1970). When species identification is desired, the capillary tubes can be broken and the buffy coat spread on a microscope slide for examination according to Murray (Murray *et al.* 1977).

The mini Anion Exchange Centrifugation Technique (mAECT) separates trypanosomes from blood cells on an anion exchange chromatography gel based on their differential surface charge in function of pH and ionic strength of the gel equilibration buffer. The buffer negatively charges the host blood cells which are subsequently adsorbed onto the anion-exchange column, while the neutral or positively charged trypanosomes are eluted, retaining their viability (Lanham & Godfrey 1970; Lumsden *et al.* 1979). Trypanosomes that are eluted from the gel are taken up in a clear glass or plastic collector tube that is centrifuged at low speed (about 1,800 g) for 10 minutes

to concentrate the parasites at the bottom of the tube where the trypanosomes can be microscopically observed under low magnification (10x10). Given that surface charges differ between species of Salivarian trypanosomes, and that the negative charge on erythrocytes also varies with mammalian species, the mAECT buffer has to be adapted in function of the host and the expected trypanosome species (Lanham & Godfrey 1970; Seaman & Uhlenbruck 1963). An improved model of mAECT column and collector tube has an analytical sensitivity of <30 trypanosomes/ml), is robust and avoids the need to mount the collector tube in water for microscopic examination (Büscher *et al.* 2009). mAECT is usually conducted on 0.5 ml of blood but prior centrifugation of a larger volume of blood (up to 5 ml) and loading the buffy coat on the mAECT column can lower the detection limit to 10 trypanosomes⁄ml (Camara *et al.* 2010). The mAECT is the most sensitive parasite detection technique with an analytical sensitivity that is similar or higher than of most molecular diagnostics for African trypanosomiasis but works only well on *T. brucei*, *T. evansi* and *T. equiperdum*. It is less performant for *T. vivax* and *T. congolense* (Büscher 2014).

9.3. Antigen detection serological tests

Attempts to develop antigen detection tests for African trypanosomoses have been undertaken by many research groups but none has resulted in a diagnostic test (Liu & Pearson 1987; Nantulya & Lindqvist 1989; Olaho-Mukani *et al.* 1993; Kashiwazaki *et al.* 1994; Nantulya 1994). The major obstacles in the development of antigen detecting tests are: 1° universal VSG capturing antibodies are difficult to design, 2° concentrations of circulating parasite antigens are too low to be detectable with the current diagnostic test formats and 3° the host immune system generates antibodies against most antigens, causing the formation of immune complexes and hampering the capturing of these antigens.

Nanobodies (Nbs) are single-domain heavy chain camel antibodies with a molecular weight of 15 kDa and have high affinity for their targets and the ability to recognize cryptic epitopes that are not easily accessed by classical antibodies. They are expected to bring new insight in development of antigen detecting diagnostic tools (Büscher 2014; Magez & Radwanska 2009). Anti-VSG Nbs were generated that recognize *T. evansi* (Saerens *et al.* 2008) and Nbs developed against the paraflagellar rod protein of *T. evansi* recognized homologues in *T. brucei, T. congolense* and *T. vivax*, indicating their potential use for the development of a diagnostic tool for AAT (Obishakin *et al.* 2014).

9.4. Antibody detecting serological tests

All infections with pathogenic trypanosomes induce high levels of antibodies, both specific and non-specific. As a consequence, detection of trypanosome specific antibodies in a host can be exploited for diagnostic purposes. Although very useful, serodiagnosis based on antibody detection has some shortcomings, for example it cannot differentiate between present and past

infection because trypanosome-specific antibodies can remain detectable in the circulation for months or even years after cure. Also, antibodies become detectable only after a couple of weeks of infection. Furthermore, antibodies due to other infections may cross react with trypanosome derived antigens used in a serodiagnostic test (Büscher 2014; Desquesnes *et al.* 2007; Nguyen *et al.* 2014; Van den Bossche *et al.* 2000). The most successful antibody detection tests for surra are based on a particular VAT of *T. evansi*, RoTat 1.2. A VAT is defined by very specific epitopes of the VSG that are exposed on the surface of a living trypanosome. Some VATs, like RoTat 1.2 are expressed early during infection by the majority if not all the different strains within a trypanosome taxon and therefore are called predominant (Büscher 2014; Van Meirvenne *et al.* 1995). The VAT RoTat 1.2 has been cloned from a *T. evansi* strain, isolated in 1982 from a water buffalo in Indonesia (Bajyana Songa & Hamers 1988). The RoTaT 1.2 VAT is expressed by most *T. evansi* strains collected from over the world, except by *T. evansi* type B and some *T. evansi* type A strains that do not express the RoTat 1.2 VSG gene (Claes *et al.* 2004; Ngaira *et al.* 2005; Ngaira *et al.* 2004; Claes *et al.* 2003b). Currently, most antibody detection tests make use of native antigens but there is an encouraging trend to replace these native antigens by recombinant antigens.

Card Agglutination Test for Trypanosomosis: The CATT/*T. evansi* is a direct agglutination test and is one of the OIE recommended tests for diagnosis of surra. The test makes use of fixed and Coomassie stained freeze dried whole trypanosomes of *T. evansi* VAT RoTat 1.2. Both variable and invariable surface antigens take part in the agglutination reaction (Bajyana Songa & Hamers 1988; OIE 2012). The test kit, which consists of freeze dried antigen, buffer, plastic-test cards, spatulas, positive and negative control sera and a rotator, is available from the Applied Technology and Production Unit at ITM, Antwerp (Figure 1.9). In its lyophilized form, the antigen is stable for at least two years at 2–8°C. The test can be performed on a drop of whole blood or on diluted serum or plasma. Specimens are tested by mixing 25 µl of sample with one drop of reconstituted antigen on a test zone of the test card. After 5 minutes rotation at 70 rpm, the result can be scored. When agglutionation is visible, the test is considered positive. CATT/*T. evansi* is suitable for detection of early as well as late infections with a high positive predictive value (OIE 2012). CATT/*T. evansi* is intended for screening of plain blood or minimally diluted plasma or serum and therefore inevitably is prone to false positive results. Its accuracy is best in situations with relatively high disease prevalence (high negative predictive value) (Büscher 2014).

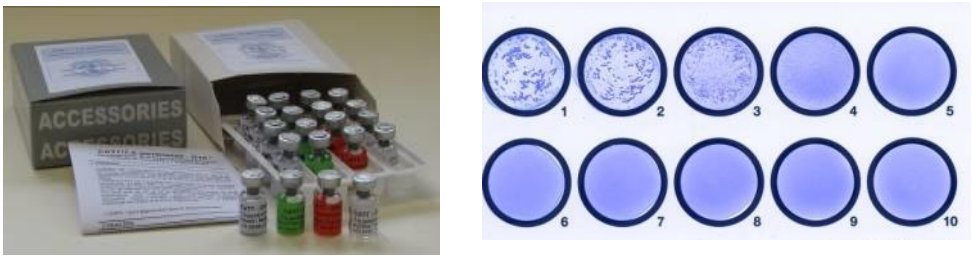

Figure 1.9: CATT/*T. evansi* kit and agglutination reaction on the plastic card.

Enzyme linked immunosorbent assay: ELISA/*T.evansi* exists in two forms that are recommended by OIE. The first uses purified RoTat 1.2 VSG (Verloo *et al.* 2000). Since ELISA is performed on highly diluted plasma or serum and since it uses a purified antigen, its intrinsic specificity is higher than CATT/*T.evansi*. ELISA is restricted to well equipped laboratories with regular supply of electricity and pure water and needs host-specific conjugates. For bovines, the use of monospecific anti-IgG conjugates is generally recommended. When specific conjugates are not available, non-specific proteins able to bind to the Fc fragment of immunoglobulins, such as protein A, can be used. Protein A conjugate has been validated for use in camels and works also well with horse specimens (OIE 2012; Desquesnes *et al.* 2009). A second variant of ELISA is based on crude water soluble extract of a non-cloned strain of *T. evansi*. It has the advantage that in principle it can detect *T. evansi* infections with strains that do not express RoTat 1.2. However, it has been shown to cross-react with other infections such as *T. cruzi* and therefore is less specific than the ELISA with purified RoTat 1.2 VSG (Desquesnes *et al.* 2007).

Indirect immunofluorescent antibody test (IFAT): This test is based on the reaction of antibodies in the test specimen (blood, serum, plasma) with intact trypanosome, preferably a cloned population of *T. evansi* RoTat 1.2 grown in mice, that are fixed on a a microscope slide. Antibodies that bind to these trypanosomes are detected with a species specific fluorescently labeled conjugate (Katende *et al.* 1987). In general, monospecific anti-IgG (gamma-chain) conjugates give the most specific results (OIE 2012). Compared with the CATT, IFAT is more sensitive but specificity is lower (Luckins 1992; Dia *et al.* 1997b). It requires a fluorescence microscope and usually, the antigen preparation is not standardized (Nantulya 1990). Reproducibility of the test has sometimes been questioned. For these reasons, ELISA is a more advisable laboratory technique (Ferenc *et al.* 1990).

Latex agglutination test: The LATEX/*T. evansi* is a rapid indirect agglutination test in which the antigen consists of soluble purified RoTat 1.2 VSG covalently coupled to microscopic latex particles. The reagent is stabilized by lyophilisation and rehydrated with deionized water before use. As for CATT/*T.evansi*, the test is performed by mixing one drop of antigen with 25 µl of diluted plasma or serum on a plastic card and let it react for 5 min on a rotator at 70 rpm (Verloo *et al.* 2000).

Immunochromatographic test (ICT): ICTs, usually in the form of a lateral flow device, have some major advantages over other serological tests formats that make them genuine rapid diagnostic tests (RDT) applicable in field conditions without any laboratory facility. RDTs are supposed to fulfil the ASSURED criteria: Affordable, Sensitive, Specific, User-friendly (minimal manipulations, easy readout), Rapid and Robust (readout within <30 min, stable at ambient temperature), Equipment free and Deliverable to the end user (Peeling *et al.* 2006; Mabey *et al.* 2004). Another advantage of lateral flow tests that is of particular importance in AAT, is their design that allows to detect antibodies without the need of host species-specific or Ig class-specific conjugates. Thus, as for direct agglutination and inhibition test formats, the same device

can be used for testing bovine, camel, horse, etc. (Büscher 2014). For surra, only one ICT is commercialized, the Surra Sero-*K*-Set (Coris BioConcept, Belgium) (Rogé *et al.* 2013). A typical antibody detection lateral flow ICT contains a sample application pad (made of cellulose and/or glass fiber that may or may not function as a filter to retain the blood cells), a conjugate pad (the place where labeled antigen-conjugate is dispensed), a nitrocellulose membrane with antigen and antibody spotted on respectively the test and control line and an adsorbent pad (works as sink at the end of the strip) (Sharma *et al.* 2015). As an example, the architecture of the Surra Sero-*K*-Set is represented in Figure 1.10.

Immune trypanolysis test (TL): This test requires the growth of trypanosomes in rodents, is costly and is only performed at the OIE Reference Laboratory for Surra at ITM, Antwerp (OIE 2012). TL makes use of living cloned populations of *T. evansi*, all expressing the same VAT RoTat 1.2. When incubated with a specimen that contains RoTat 1.2-specific antibodies and with guinea pig serum as complement source, the trypanosomes will be destroyed by antibody-mediated complement lysis (Van Meirvenne *et al.* 1995). The principle of TL is represented in Figure 1.11. A serum or plasma sample is considered positive for the presence of anti-RoTat 1.2 antibodies when at least 50% of the trypanosomes are lysed after 90 minutes incubation. TL with *T. evansi* RoTat 1.2 is considered 100% specific since the only epitopes at the surface of the living trypanosomes that are available for reaction with the host antibodies are the VAT specific epitopes. This is contrasting with CATT, LATEX, ICT, ELISA and IFAT where other, cross reacting epitopes can react with non-trypanosome specific antibodies in a test sample (Verloo *et al.* 2000). The major shortcoming of TL is that it will not detect infections with *T. evansi* type B since this type does not express RoTat 1.2 VSG.

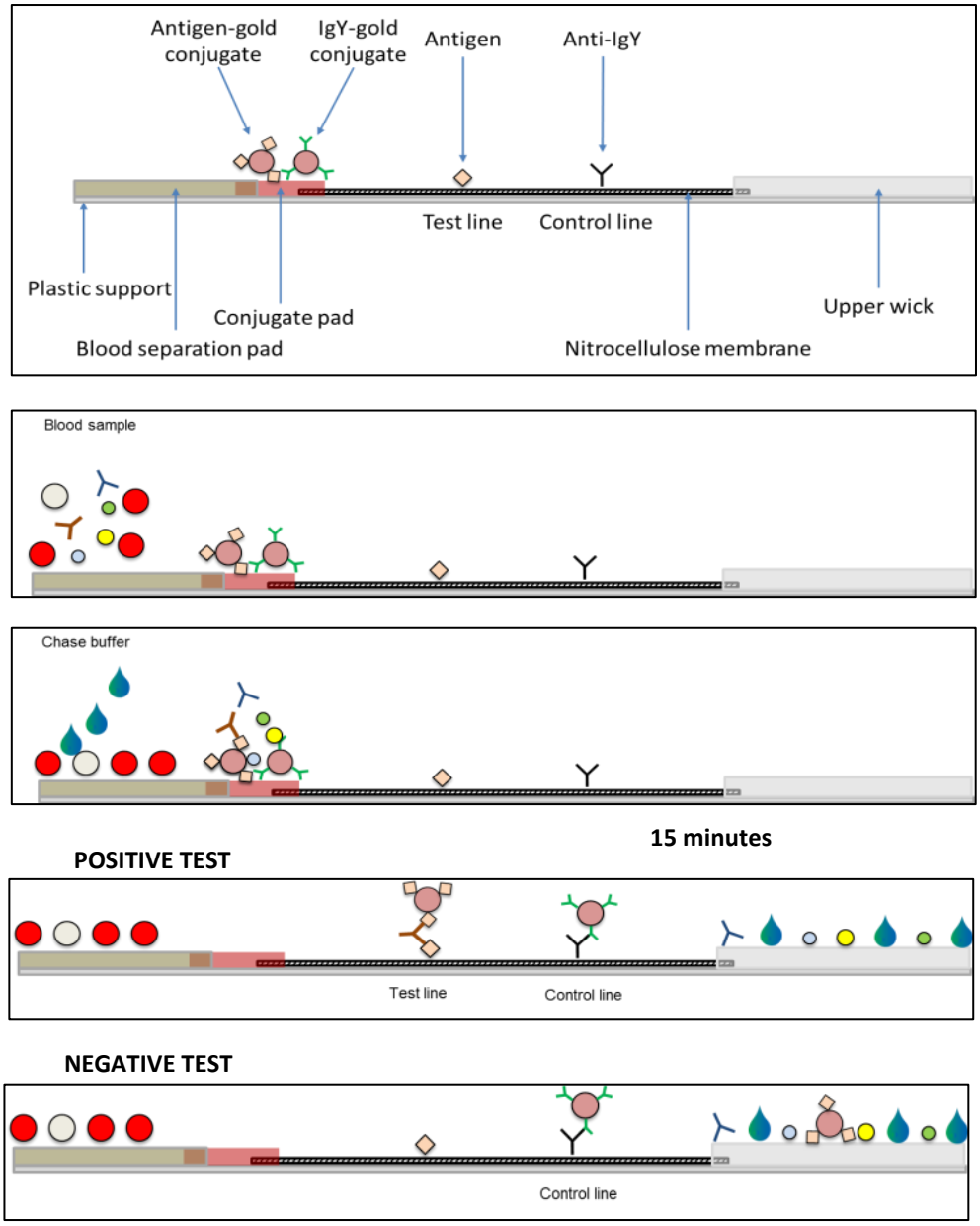

Figure 1.10: Surra Sero-*K*-SeT: architecture, adding blood and chase buffer and readout of a positive and negative sample.

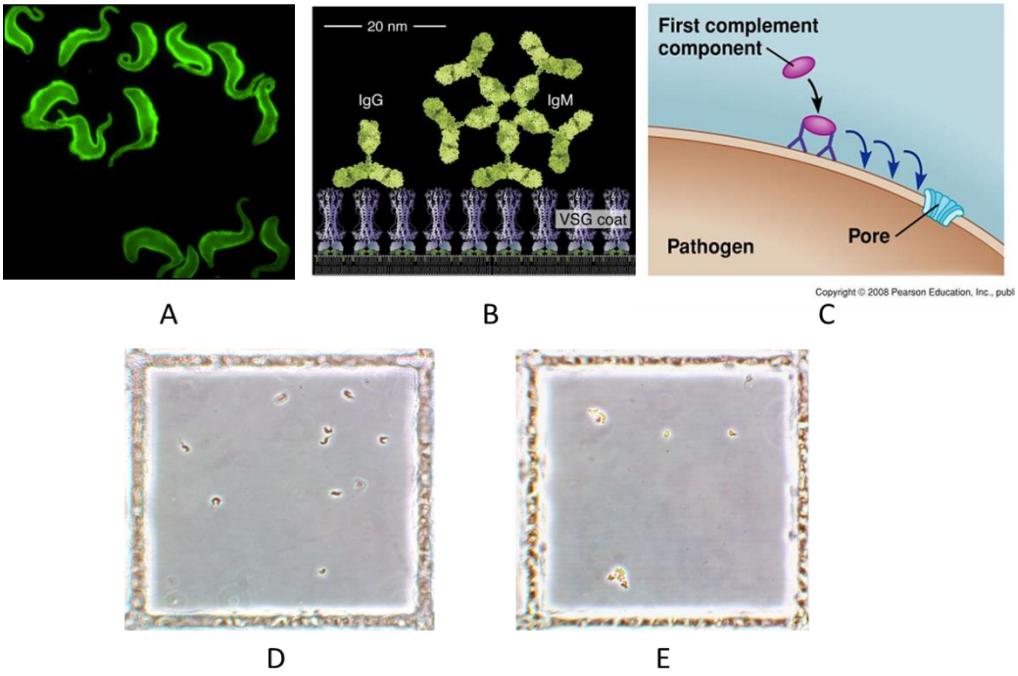

Figure 1.11: Principle of the immune trypanolysis test. (A) Live cloned *T. evansi* parasites, all expressing RoTat 1.2 VSG, are used as source of antigen; (B) RoTat 1.2VAT-specific IgG and/or IgM bind to the N-terminal epitope of the VSG coat; (C) Guinea pig serum complement is activated by the Fc part of the antibody in the immune complexes; (D) In the absence of VAT specific antibodies, trypanosomes remain intact; (E) Trypanosomes are destroyed by antibody–mediated complement lysis. Adapted from Engstler (Engstler *et al.* 2007).

9.5. Molecular diagnosis

To overcome the limitations of microscopical analysis, molecular diagnostics have been introduced. For reasons of complexity and technical requirements, molecular dignostics are not used in routine diagnosis in most AAT endemic countries. The trypanosome genomic material consists of nuclear and kinetoplastid DNA (kDNA). The nucleus contains three types of chromosomes, (a) megabase chromosomes (1–6 Mb), (b) intermediate chromosomes (200–900 kb) and (c) minichromosomes (50–150 kb) (El-Sayed *et al.* 2000). Detection of RNA is the best surrogate for detection of living parasites since DNA of dead trypanosomes can remain in the circulation for a couple of days. On the other hand, RNA rapidly degrades thus making proper specimen processing more delicate. DNA detection tests are based on the amplification of a variety of coding and non-coding sequences of genomic or kinetoplast DNA, including highly and medium repetitive sequences as well as single copy sequences (Büscher 2014). For sensitivity reasons, multicopy genes are preferred over single copy genes (Deborggraeve & Büscher 2012; Desquesnes *et al.* 2001). Non-coding sequences for which PCRs are developed are satellite DNA,

internal transcribed sequences (ITS1 and ITS2) and repetitive insertion mobile elements (RIME). The interest of these non-coding sequences lies in their usually high copy number conferring high analytical sensitivity but with the consequence that they are more prone to contamination during specimen collection or processing (Desquesnes *et al.* 2001; Thekisoe *et al.* 2007). An overview of molecular test for diagnosis of *T. evansi* infection is presented in Table 1.2.

Ribosomal DNA genes occur in multiple copies in tandem arrays. They are made up of transcriptional units (TU) separated by non-transcribed spacers (NTS). The TU consists of an 18S ribosomal subunit, an internal transcribed spacer 1 (ITS-1), a 5.8S ribosomal subunit, an ITS-2 and a 28S ribosomal subunit (Desquesnes & Dávila 2002). In animal trypanosomosis where mixed infections can occur, PCRs that allow discrimination of the trypanosome species in one single run and in the same specimen have special interest (Büscher 2014). The ITS1 with a copy number of 100-200 has a variable length depending on the *Kinetoplastida* species, but is presumed to be constant within a species (Desquesnes *et al.* 2001). Several variants of PCRs that target the ITS1 sequence do exist and recently, primers sequences have been modified to improve sensitivity (Fikru *et al.* 2012). Unfortunately, the ITS1 PCR assays are prone to non-specific amplification, particularly with bovine blood (Fikru *et al.* 2014). Therefore, a "Touchdown" PCR approach, which employs more stringent primer-template hybridisation temperatures, was introduced (Tran *et al.* 2014). In Touchdown PCR, the annealing temperature during the first PCR cycles is well above the predicted optimal annealing temperature of the primers thus favouring the amplification of the specific target sequence while in the following PCR cycles, the annealing temperature is gradually lowered to more permissive temperatures (Don *et al.* 1991; Korbie & Mattick 2008). The 18S-PCR-RFLP, a pan-trypanosome PCR followed by cleavage of the amplicons with two restriction enzymes (*Msp*1 and *Eco*571), generates fragment profiles that are characteristic for *T. congolense* (including sub group differentiation)*, T. vivax, Trypanozoon* and *T. theileri* (Geysen *et al.* 2003). The TBR1/2 PCR amplifies minichromosome satellite repetitive sequences and is considered the gold standard for detection of *Trypanozoon* DNA and allows detection of as little as 1–5 trypanosomes/ml of blood (OIE 2012; Masiga *et al.* 1992).

With the objective to develop a specific PCR test that would be able to distinguish *T. evansi* from the other members of the *Trypanozoon* subgenus, a PCR targeting the RoTat 1.2 VSG gene was proposed (Urakawa *et al.* 2001). Extended evaluation on a large collection of *Trypanozoon* taxa with this PCR showed a lower analytical sensitivity of 10 trypanosomes per ml of blood. With this PCR, Claes and co-workers were able to show that most *T. equiperdum* isolates, except BoTat 1.1 and OVI, are in fact misclassified *T. evansi* strains (Claes *et al.* 2004). Since the RoTat 1.2 gene is absent *T. evansi* type B, the RoTat 1.2 specific PCR will not be able to detect infections with this taxon (Ngaira *et al.* 2004; Ngaira *et al.* 2005; Claes *et al.* 2004). For diagnosis and identification of *T. evansi* type B infections, primers targeting type B specific minicircle kDNA sequences and JN 2118Hu VSG have been descried (Njiru *et al.* 2006; Ngaira *et al.* 2005). As an alternative to the complex PCR tests requiring temperature cycling, the loop-mediated isothermal amplification (LAMP) of DNA has been introduced (Njiru 2012). For diagnosis of AAT, LAMP tests targeting VSG

RoTat 1.2, VSG JN 2118Hu and RIME have been developed (Njiru *et al.* 2008; Njiru *et al.* 2010; Thekisoe *et al.* 2007; Kuboki *et al.* 2003).

The diagnostic sensitivity of a PCR is dependent on the initial amount of template DNA and is therefore proportional to the parasitaemia in a blood or tissue specimen (OIE 2012). Moreover, results obtained in molecular diagnostics are influenced by techniques for specimen collection, storage and DNA extraction. To overcome the usual challenges of low parasitaemia in AAT, collection of larger volumes of blood (0.2-0.8 ml) or of the buffy coat obtained after centrifugation of blood and stored in DNA stabilisation buffers containing guanidinium hydrochloride and EDTA, is recommended. These buffers allow to store the specimens for months at ambient temperature and are compatible with most of the commercial DNA extraction kits (Deborggraeve *et al.* 2011; De Winne *et al.* 2014).

For reference molecular diagnosis, it is necessary to check the quality of the DNA extracted from a specimen and the absence of polymerase inhibitors. Therefore, trypanosome specific PCRs should be accompanied by a mammalian cytochrome B-specific PCR (Kocher *et al.* 1989).

Table 1.2: Molecular tests for diagnosis of *T. evansi* infection in animals.

Specificity	**Target**	**Primers**	**Primer sequence (5'-3')**	**Amplicon size (bp)**	**Reference**
Trypanosoma	ITS1	Kin 1 Kin 2	GCG TTC AAA GAT TGG GCA AT CGC CCG AAA GTT CAC C	T*rypanozoon* (535), *T. vivax* (307), *T. congolense* (671, 754), *T. theileri* (454),	(Desquesnes *et al.* 2001)
Trypanosoma	ITS1	forward reverse	TGTAGGTGAACCTGCAGCTGGATC CCAAGTCATCCATCGCGACACGTT	*Trypanozoon* (450), *T. vivax* (150), *T. congolense Savannah* (650), *T. theileiri* (350)	(Fikru *et al.* 2012)
Trypanosoma	ITS1	forward reverse	TGT AGG TGA ACC TGC AGC TGGATCT CCA AGT CAT CCA TCG CGA CAC GT	*Trypanozoon* (391-393), *T. vivax* (165), *T. congolense Savanna* (612), *T. theileri* (300)	(Tran *et al.* 2014)
Trypanosoma	Ssu-rDNA	18ST nF2 18ST nF3 18ST nR2	CAACGATGACACCCATGAATTGGGGA TGCGCGACCAATAATTGCAATAC GTGTCTTGTTCTCACTGACATTGTAGTG	Restriction pattern	(Geysen *et al.* 2003)
Trypanozoon	Minichromosome satellite repetitive sequence	TBR1 TBR2	GAATATTAAACAATGCGCAG CCATTTATTAGCTTTGTTGC	164	(Masiga *et al.* 1992)
T. evansi type A	RoTat 1.2 VSG	ILO7957 ILO8091	GCC ACC ACG GCG AAA GAC TAA TCA GTG TGG TGT GC	488	(Urakawa *et al.* 2001)
T. evansi type A	RoTat 1.2 VSG	forward reverse	GCG GGG TGT TTA AAG CAA TA ATT AGT GCT GCG TGT GTT CG	205	(Claes *et al.* 2004)
T. evansi type A	RoTat 1.2 VSG	FIP BIP F3 B3	TTCGATCGCTGCGAAGTGCGTCTGGAA GCCATTGTGCG AAGCTCTTGATTTACGCGGCGGGCTGC TAACCCTCTTGCTG GCCGCCAATGTAGCTCTT CCGCTGCTCGTATGTGTC	200	(Thekisoe *et al.* 2007)
T. evansi type B	minicircle	EVAB1 EVAB2	CACAGTCCGAGAGATAGAG CTGTACTCTACATCTACCTC	436	(Njiru *et al.* 2006)
T. evansi type B	VSG JN 2118Hu	forward reverse	TTCTACCAACTGACGGAGCG TAGCTCCGGATGCATCGGT	273	(Ngaira *et al.* 2005)
T. evansi type B	VSG JN 2118Hu	TeB-F3 TeB-B3 TeB-biotin FIP TeB-BIP TeB-LF TeB-LB TeB-FITC	CCAATCAAAGACGAGCGG TGGTTTGTGAGGCCGCAG CGGATGCATCGGTGATGCAATCACTAC TGCATCAAGGGAAGC ATCCAGCACCTCGGAACAGCTCTCGGC AACCAGATCGG GTTCACGTGCCTCCGCTTC ACGTAGCGGGAAAATACGC CTATCCTAAAAGAAGCTGGAG	171	(Njiru *et al.* 2010)

10. Recombinant expression of *T. evansi* derived antigens for diagnostic purposes

As mentioned above, there is a successful tendency to replace native antigens by recombinant antigens in the serological diagnostic tests for surra. Several expression systems can be used for the production of recombinant proteins. Selection of the expression system depends mainly on considerations of development and running cost, yield, and easiness of downstream processing, and necessity of correct glycosylation (Anné *et al.* 2014). The hosts commonly been used for the expression of *T. evansi* proteins are *Escherichia coli, Pichia pastoris,* and *Spodoptera frugiperda* (Nguyen *et al.* 2014; Tran *et al.* 2009; Rogé *et al.* 2013; Lejon *et al.* 2005; Urakawa *et al.* 2001).

10.1. *Escherichia coli* (*E. coli*)

The main advantages of using *E. coli* as heterologous protein expression host are its fast growth kinetics with easily achievable high cell density cultures, its low requirements for the growth median and its fast and easy constituve transformation with exogenous DNA (Rosano & Ceccarelli 2014). The Origami *E. coli* strains (Novagene, USA) have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes which enhance disulfide bond formation. The Rosetta and Rosetta 2 *E. coli* strains are designed to enhance expression of eukaryotic proteins that contain codons rarely used in *E. coli*. The original Rosetta strain supplies tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and GGA on a compatible chloramphenicol-resistant plasmid, pRARE. The Rosetta 2 strain supplies a seventh rare codon (CGG). The tRNA genes are driven by their native promoters (Novagen, USA).

In most *E. coli* hosts, heterologous proteins are usually produced in the periplasm, which facilitates isolation of the proteins from this compartment and, more importantly, in the oxidizing environment of the periplasm the disulfide bond formation (Dsb) system catalyzes the formation of disulfide bonds. In order to reach the periplasm, the heterologous proteins are equipped with an N-terminal signal sequence that guides them to the Sec-translocon, which is a protein-conducting channel in the cytoplasmic membrane (Schlegel *et al.* 2013). Moreover, the pET-22b(+) expression vector has a signal sequence (pelB leader) for secretion of expressed protein to the periplasm; a C-terminal His-Tag sequence for affinity purification; f1 origin of replication and confers ampicillin resistance to host cells (Novagen, USA). The main drawbacks of using *E. coli* as expression system are the absence of post-translational modifications, like N-linked glycosylation, that may play a role in stability, functionality and antigenicity of eukaryotic proteins. Furthermore, the bacteria is enveloped by an outer membrane consisting of lipopolysaccharides which is a source of endotoxins and hinders the secretion of proteins into the extracellular environment often leading to accumulation of the expressed proteins in the cytoplasm. In these insoluble aggregates known as inclusion bodies, proteins are in an inactive conformation and it is often difficult to recover them in an active form (Anné *et al.* 2014). Several *T. evansi* derived

proteins have been successfully expressed in *E. coli.* For example, the N-terminal extracellular domain of ISG75 from LiTat 1.3 *T.b. gambiense* was expressed in a *E. coli* Origami strain. The recombinant double-tagged (streptavidine and histidine) ISG75 fragment was purified by two-step affinity chromatography but with low yield (0.5 mg/l). The purified recombinant showed definite potential as diagnostic antigen for camels in an ELISA system (Tran *et al.* 2008; Tran *et al.* 2009). Another example is *Te*GM6-4r that was successfully expressed in a *E. coli* DH5 strain. The purified recombinant protein was tested in ELISA and showed promising diagnostic accuracy when tested on *T. evansi* infected water buffaloes (Nguyen *et al.* 2014). Also, a VSG fragment, expressed in *E. coli*, showed diagnostic potential in ELISA (Sengupta *et al.* 2012).

10.2. *Pichia pastoris*

The methylotrophic yeast *Pichia (P.) pastoris*, is suited to heterologous protein expression of either prokaryotic or eukaryotic origins. The advantages of its use are ease of genetic manipulation with constitutive expression, high efficiency DNA transformation, high levels of protein expression at intra or extracellular level, and the ability to perform eukaryotic protein modifications such as glycosylation and disulphide bond formation (Macauley-Patrick *et al.* 2005). However, glycoproteins produced in *P. pastoris* contain high mannose glycan structures. Those structures can hamper downstream processing, might be immunogenic and can cause rapid clearance from the circulation (Vervecken *et al.* 2004). The hypermannosylation can be prevented by using the M5 strain of *P. pastoris*, that has an engineered N-glycosylation pathway resulting in homogenous Man5GlcNAc2 N-glycosylation which resembles the predominant Man9-5GlcNAc2 oligomannose structures in *T. brucei* (Vervecken et al. 2004; Rogé et al. 2013; Puxbaum et al. 2015). The N-terminal part of VSG RoTat 1.2 was recombinantly expressed in the yeast *P. pastoris* with yields up to 20 mg/l of cell culture and high reproducibility and definite diagnostic potential in ELISA and ICT format (Rogé *et al.* 2013). Similarly, the N-terminal fragment of ISG75 from *T. b. gambiense* LiTat 1.3 was expressed in a *P. pastoris* M5 strain. The yield was 10 mg/l cell culture. Unfortunately, several shorter degradation products were observed (Rogé *et al.* 2013). This protein degradation didn't occur when the same gene was recombinantly expressed in *E. coli* (Tran *et al.* 2008). The recombinant ISG75 fragment purified from *P. pastoris* showed good diagnostic potential in experimentally infected goats but not in naturally infected and control camels thus contradicting the results obtained with a similar recombinant ISG75 fragment expressed in *E. coli* (Tran *et al.* 2008; Tran *et al.* 2009; Rogé *et al.* 2013). This example clearly illustrates the effect of the expression system on the eventual diagnostic potential of an antigen.

10.3. *Spodoptera frugiperda*

Insect cells like the one from *Spodoptera frugiperda*, can provide the desired folding and disulfide bond formation, as well as oligomerization of a recombinant protein. The disadvantage of classical *S. frugiperda* expression systems is that they are only transiently transfected by a virus, *in casu* a bacculovirus, that contains the gene of interest. The gene of interest is integrated

into a nonessential region of the viral genome by homologous recombination, hence large cDNA inserts (up to 15 kb) can be accommodated and recombinant virus can be amplified to high titers, thereby leading to high-level protein production after infection of insect cells (Ikonomou *et al.* 2003). The *Spodoptera (S.) frugiperda* insect cells have been used for successful production of recombinant N-terminal fragments of RoTat 1.2 VSG of *T. evansi* (Urakawa *et al.* 2001; Lejon *et al.* 2005). With the purified recombinant RoTat 1.2 VSG fragment, an ELISA and a latex agglutination test were prepared and both showed acceptable diagnostic accuracy. Unfotunately, the expression of the recombinant VSG RoTat 1.2 in *S. frugiperda* was poorly reproducible and attempts to scaling up the production were not successful (Lejon *et al.* 2005).

10.4. *Leishmania tarentolae*

Recombinant antigens expressed in *E. coli* are not glycosylated and therefore may miss some critical epitopes with diagnostic potential that may be present on glycosylated and correctly folded native glycoproteins. In contrast, *Leishmania (L.) tarentolae* can yield glycosylated recombinants that can be engineered to be secreted into the culture medium. *L. tarentolae* is a eukaryotic flagellated unicellular parasite, closely related to *T. brucei,* and used as a host for recombinant protein expression (Breitling *et al.* 2002). Its ability to produce mammalian like complex type N-glycosylation, easy genetic manipulation, straightforward adaptation to large-scale production, and minimal nutrition requirement makes it an excellent expression host (Basile & Peticca 2009; Fritsche *et al.* 2007). *L. tarentolae* allows higher levels than other eukaryotes (Hacker *et al.* 2009; Jenkins *et al.* 2009; Klatt & Konthur 2012). A major advantage of using this expression system is that recombinants can either be expressed intracellularly or secreted into the culture medium and because *L. tarentolae* secretes only low levels of endogenous proteins and its culture medium. This separation of the heterologous protein from the bulk of cellular proteins facilitates purification. Recently, the extracellular domains of ISG65 and VSG LiTat 1.3 and VSG LiTat 1.5 were successfully expressed in *L. tarentolae* and showed exceptionally high diagnostic accuracy when tested on a panel of reference sera of *T.b. gambiense* patients and endemic controls (Rooney *et al.* 2015).

Objectives and study design

1. Background

In Ethiopia, both cyclically and mechanically transmitted pathogenic *Trypanosoma* species occur. *T. evansi* infection is widely distributed across the six agro-climatic zones, mainly coinciding with the distribution of camels (Dagnatchew 1982; Abebe & Yilma 1996; Abebe 2005). In developing countries including Ethiopia, diagnosis of NTTAT makes use of poorly sensitive and non-specific diagnostic techniques such as clinical examination and direct microscopic examination of blood. Epidemiological studies using *T. vivax* and *T. evansi* specific markers have only recently been undertaken in Ethiopia (Fikru *et al.* 2015; Hagos *et al.* 2009; Hagos *et al.* 2010b). Poorly sensitive and specific diagnostics inevitabily lead to underestimation or overestimation of the burden of NTTAT and of its impact on livestock production in the country.

Thus, part of this PhD study is focused on the epidemiology of NTTAT due to T. evansi and T. vivax in domestic animals from selected districts of Tigray and Afar regions, both considered as tsetse free areas in Ethiopia.

T. evansi strains from Ethiopia are typically lacking in the cryocollection of the World Animal OIE Reference Center for surra at ITM and elsewhere. Furthermore, there are no published reports on drug sentivity profiles of *T. evansi* strains in Ethiopia.

Thus, part of this PhD study is focused on isolation, phenotypic and genotypic characterization, in vitro adaptation and determination of drug sensitivity pattern of T. evansi isolates from Ethiopia.

Accurate diagnosis is crucial for early detection of infections, evaluation of treatment efficacy, better understanding of the epidemiology of the disease and for designing appropriate control strategies. Conclusive evidence of *T. evansi* infection relies on detection of the parasite or its DNA in the body fluids of infected animals. Unfortunately, parasitological techniques cannot always detect ongoing infections as the level of parasitaemia is often low and fluctuating, particularly during the chronic stage of the disease. Molecular diagnostic tests are highly appreciated for surveillance and research purposes. Sensitive DNA detection tests such as the ITS1 and TBR PCRs are not *T. evansi* specific and will detect also *T. brucei* and *T. equiperdum* (Desquesnes *et al.* 2001; Masiga & Gibson 1990). *T. evansi* type A specific RoTat 1.2 PCR and *T. evansi* type B specific kDNA minicircle PCR are available but the latter will not be able to detect akinetoplast *T. evansi* strains that may occur naturally (Claes *et al.* 2004; Njiru *et al.* 2006). Moreover, the diagnostic sensitivity of molecular diagnostics is also hampered by the often low and fluctuating parasitaemia in *T. evansi* infections. For infection with undetectable parasite loads, suspicion of surra can be obtained with antibody detection tests.

For antibody detection, the most widely used OIE recommended test is the CATT/*T. evansi*. The antigen in this test consists mainly of a *T. evansi* specific VSG as antigen (*in casu* RoTat 1.2) (OIE 2012). Production of CATT/*T. evansi* requires mass culture of *T. evansi* in laboratory rodents. The use of whole trypanosomes and complex antigen molecules in the CATT and similar antibody

tests gives rise to cross-reactivity with non *T. evansi* specific antibodies resulting in decreased test specificity (Büscher 2014; Desquesnes *et al.* 2007). To overcome these challenges, ITM and Coris BioConcept (Gembloux, Belgium) developed the Surra Sero *K*-SeT, that makes use of recombinant VSG RoTat 1.2 expressed in *P. pastoris.*

Thus, part of this study is focused on the diagnostic evaluation of the Surra Sero-K- SeT on large serum collections.

2. General objective

The general objective was to study the epidemiology, diversity and alternative diagnostics for NTTAT in Ethiopia

3. Specific objectives

- Assess the epidemiology of NTTAT in domestic animals from Tigray and Afar regions of Ethiopia *(Chapter 3)*;
- Isolate, and phenotypically and genotypically characterize Ethiopian *T. evansi* stocks *(Chapter 4)*;
- Adapt to *in vitro* culture and determine *in vitro* drug senstivity profiles of Ethiopian *T. evansi* stocks *(Chapter 4)*;
- Evaluate the diagnostic accuracy of Surra Sero-*K*-SeT, the first RDT for serodiagnosis of surra *(Chapter 5)*.

4. Study design

This study consists of both field and laboratory based activities that were conducted partly in Ethiopia and partly in Belgium. Field activities took place in 4 districts of Tigray region with crop-livestock mixed agriculture and in 5 districts of Afar region, in one of the main pastoral areas of Ethiopia. After ethical clearance of the study protocols, blood specimens were collected from the jugular vein of 1811 randomly selected animals (754 dromedary camels, 493 cattle, 264 goats, 181 sheep, 84 donkeys, 25 horses, and 10 mules). Parasitological examination and PCV recording was performed by mHCT of 4 capillary tubes. Animals with detectable trypanosomes were treated with appropriate doses of cymelarsan, DA or ISM. The rest of the blood was centrifuged to collect plasma for antibody detection with CATT/*T. evansi*. Part of the plasma was spotted on Whatman 4 filter paper for testing in RoTat 1.2 TL, the gold standard test for serodiagnosis of surra (Bajyana Songa & Hamers 1988; Verloo *et al.* 2001; OIE 2012). The corresponding buffy coat specimens were preserved in guanidium-EDTA buffer for molecular analysis. Furthermore, of all parasitologically positive animals, a copy of the buffy coat was cryopreserved in liquid nitrogen for later isolation of the trypanosome strain by inoculation of Swiss albino mice at the College of Veterinary Medicine, Mekelle University (Pyana *et al.* 2011). Stabilate copies were imported to ITM in Belgium and expanded in mice for genetic characterisation, *in vitro* adaptation and drug

sensitivity profiling. DNA extraction from the stored buffy coats followed by testing with species-specific PCRs was also conducted in ITM. RoTat 1.2 PCR, EVAB PCR and *Tv*PRAC PCR were used to identify respectively *T. evansi* type A, *T. evansi* type B and *T. vivax* (Claes *et al.* 2004; Njiru *et al.* 2006; Fikru *et al.* 2014). To addresses some limitations of current molecular markers for *T. evansi* genotyping, the polymorphism within the F1-ATP synthase γ subunit gene was investigated. For the evaluation of the Surra Sero-*K*-SeT, a large collection of sera originating from diverse domestic animal species and from diverse geographical origin was available in the cryobank of the OIE Reference Center for Surra at ITM.

The results of the PhD study are presented as separate chapters corresponding with published manuscripts (Chapters 3 to 5), followed by a general discussion and perspectives for further research (Chapter 6).

Epidemiology of *Trypanosoma evansi* and *Trypanosoma vivax* in domestic animals from selected districts of Tigray and Afar regions, Northern Ethiopia

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Author contributions: HB and PB conceived this study, generated, analysed and interpreted the data and prepared the manuscript. BG and GT contributed for the conception of the study and provided technical and infrastructural support to the field and laboratory work in Ethiopia. HA, RF, KW and AT participated in the field and part of the laboratory work. DB and SM performed the statistical analysis on the data. DT designed the map of the study areas. All authors revised and approved the final manuscript.

1. Abstract

African animal trypanosomosis (AAT), transmitted cyclically by tsetse flies or mechanically by other biting flies, cause serious inflictions to livestock health. This study investigates the extent of non-tsetse transmitted animal trypanosomosis (NTTAT) by *Trypanosoma (T.) evansi* and *T. vivax* in domestic animals in the tsetse-free regions of Northern Ethiopia, Afar and Tigray.

A cross sectional study was conducted on 754 dromedary camels, 493 cattle, 264 goats, 181 sheep, 84 donkeys, 25 horses and 10 mules. Microhaematocrit centrifugation technique was used as parasitological test. Plasma was collected for serodiagnosis with CATT/*T. evansi* and RoTat 1.2 immune trypanolysis (TL) while buffy coat specimens were collected for molecular diagnosis with *T. evansi* type A specific RoTat 1.2 PCR, *T. evansi* type B specific EVAB PCR and *T. vivax* specific *Tv*PRAC PCR.

The parasitological prevalence was 4.7% in Tigray and 2.7% in Afar and significantly higher ($z=2.53$, $p=0.011$) in cattle (7.3%) than in the other hosts. Seroprevalence in CATT/*T. evansi* was 24.6% in Tigray and 13.9% in Afar and was significantly higher ($z=9.39$, $p<0.001$) in cattle (37.3%) than in the other hosts. On the other hand, seroprevalence assessed by TL was only 1.9% suggesting cross reaction of CATT/*T. evansi* with *T. vivax* or other trypanosome infections. Molecular prevalence of *T. evansi* type A was 8.0% in Tigray and in Afar and varied from 28.0% in horses to 2.2% in sheep. It was also significantly higher ($p<0.001$) in camel (11.7 %) than in cattle (6.1%), donkey (6%), goat (3.8%), and sheep (2.2%). Four camels were positive for *T. evansi* type B. Molecular prevalence of *T. vivax* was 3.0% and was similar in Tigray and Afar. It didn't differ significantly among the host species except that it was not detected in horses and mules.

NTTAT caused by *T. vivax* and *T. evansi*, is an important threat to animal health in Tigray and Afar. For the first time, we confirm the presence of *T. evansi* type B in Ethiopian camels. Unexplained results obtained with the current diagnostic tests in bovines warrant particular efforts to isolate and characterise trypanosome strains that circulate in Northern Ethiopia.

2. Introduction

African trypanosomosis is one of the most important animal diseases encountered in all agro-ecological zones of the country and hinders the efforts made for food self-sufficiency (Abebe 2005). African trypanosomosis is a general term for infections in many different hosts (man and his domestic animals and wild animals) caused by various trypanosome species with *Trypanosoma (T.) brucei*, *T. congolense, T. vivax, T. evansi* and *T. equiperdum* as the most important ones (Hoare 1972). African animal trypanosomoses (AAT) cause serious inflictions to the health of livestock ranging from anaemia, loss of condition and emaciation, abortion, death etc. (Da Silva *et al.* 2011; Van den Bossche 2000; Da Silva *et al.* 2010; Da Silva *et al.* 2010; Losos 1986; Gutiérrez *et al.* 2005; Löhr *et al.* 1986). The trypanosomes responsible for AAT in Ethiopia are *T. vivax*, *T. congolense*, *T. brucei*, *T. evansi* and *T. equiperdum* (Dagnatchew 1982).

T. congolense and *T. brucei* are exclusively found in the tsetse-infested areas of Ethiopia while *T. evansi* and *T. equiperdum* occur in the tsetse-free areas. *T. vivax* can be found in both tsetse-infested and tsetse-free areas except in the highlands, which are >2500 meter above sea level (Dagnatchew 1982; Abebe & Yilma 1996).

T. evansi has multiple means of transmission of which mechanical transmission by biting insects is the most important in camels and other large animals. Other transmission routes such as the bite of vampire bats and oral transmission in carnivores has been documented (Hoare 1972; Raina *et al.* 1985; Sinha *et al.* 1971).

In Ethiopia, *T. evansi* is widely distributed across the six agro-climatic zones and mainly coincides with the distribution of camels (Langridge 1976). Trypanosomosis due to *T. evansi* (surra) is the number one protozoan disease of camels. Horses are also very susceptible. Infected camels and equines may die within 3 months. Moreover, cattle, buffalo, pigs, goat and sheep infected with *T. evansi* suffer from immunosuppression, resulting in increased susceptibility to other diseases or in vaccination failure (Desquesnes *et al.* 2013b; Desquesnes *et al.* 2013a; Gutiérrez *et al.* 2006b). For example, experimental infections in buffalo and pigs have shown reduced cellular and humoral responses after vaccination against classical swine fever and *Pasteurella multicoda* in *T. evansi* infected animals compared to uninfected animals (Holland *et al.* 2003; Singla *et al.* 2010; Holland *et al.* 2001).

Despite the considerable number of epidemiological studies carried out in Ethiopia on cattle and camel trypanosomosis in parts of Southern Nations, Nationalities, and Peoples' Region (SNNPR), and in Oromiya and Amhara regions, information from Tigray and pastoral areas of Afar, belonging to the tsetse-free areas of Ethiopia, is scanty (Sinshaw *et al.* 2006; Codjia *et al.* 1993; Rowlands *et al.* 1993; Hagos *et al.* 2010b; Miruk *et al.* 2008; Cherenet *et al.* 2006; Fikru *et al.* 2012; Hagos *et al.* 2009). In addition, due to limited logistic resources and poor diagnostic facilities, the exact burden and socioeconomic impact of AAT is probably underestimated and information on prevailing trypanosome species and affected hosts remains inaccurate and

fragmented (Aradaib & Majid 2006; Büscher 2001; Fikru *et al.* 2012). Therefore, this study was designed to investigate the distribution of *T. evansi* and *T. vivax* in selected districts of Tigray and in pastoral areas of Afar.

Diagnosis of AAT is often based on clinical suspicion. Parasite detection is cumbersome in many cases where only low numbers of trypanosomes circulate in the host body fluids (Büscher 2001). Techniques for concentration of the trypanosomes by centrifugation of a blood specimen can be applied. After centrifugation of some blood in a capillary tube, the trypanosomes can be detected directly under the microscope at the level of the white blood cell layer (the buffy coat) (Woo 1969). Where differential diagnosis is needed, the capillary tube can be broken and the buffy coat spread on a microscope slide for examination according to (Murray *et al.* 1977). A more sensitive technique is the mini Anion Exchange Centrifugation Technique (mAECT) but the technique works best with *T. brucei* and *T. evansi* and has poor diagnostic potential for *T. congolense* and *T. vivax* (Lanham & Godfrey 1970; Lumsden *et al.* 1979; Zillmann *et al.* 1996; Büscher *et al.* 2009).

As an alternative to parasitological diagnosis, molecular diagnostic tests have been developed. For the diagnosis of surra, the PCR RoTat 1.2 and Q-PCR RoTat 1.2 are specific for *T. evansi* type A and PCR EVAB is specific for *T. evansi* type B (Njiru *et al.* 2006; Claes *et al.* 2004; Konnai *et al.* 2009). For the molecular diagnosis of *T. vivax*, the ITS-1 PCR and proline racemase PCR (*Tv*PRAC PCR) can be employed (Desquesnes *et al.* 2001; Fikru *et al.* 2014). Neither parasitological nor molecular tests are 100% sensitive, due to the often low number of circulating parasites.

Serological tests are able to reveal ongoing or past trypanosome infections based on antibody detection. For surra, the most specific antibody detection tests make use of the *T. evansi* specific variant surface glycoprotein (VSG) RoTat 1.2 as antigen. The CATT/*T. evansi* is such a test in the form of a direct agglutination test and is the only rapid diagnostic test for surra that is recommended by the World Organization for Animal Health (OIE 2012; Bajyana Songa & Hamers 1988). By virtue of its format as a direct agglutination test, CATT/*T. evansi* can be applied on any host species. Knowledge about the antigenic repertoires of *T. vivax* is almost nonexistent. Most antibody detection tests for *T. vivax* make use of more or less purified native antigens leaving room for aspecific reactions. In regions where *T. vivax* and *T. brucei* or *T. evansi* occur together in the same host species, it is almost impossible to identify the infecting trypanosome species at the level of circulating antibodies in the host (Büscher 2001; Camargo *et al.* 2004; Uzcanga *et al.* 2004; Uzcanga *et al.* 2002). Only recently, recombinant *T. vivax* specific antigens are being investigated for their diagnostic potential (Pillay *et al.* 2013).

The present study provides data on the epidemiology of AAT in domestic animals in two tsetse-free regions of Ethiopia making use of the diagnostic tests presented in Table 3.1.

Table 3.1: Some characteristics of the diagnostic tests used in the epidemiological survey.

Test	Accuracy /lower detection limit	Target	Reference
mHCT	500 trypanosomes/ml	Trypanosomes	(Chappuis *et al.* 2005)
CATT/*T. evansi*	sensitivity (73.8-100%), specificity (76.9-100%)	*T. evansi* type A	(Rogé *et al.* 2014)
TL	sensitivity (100%) specificity (100%)	*T. evansi* type A	(Verloo *et al.* 2000)
RoTat 1.2 PCR	10 trypanosomes/ml	*T. evansi* type A	(Claes *et al.* 2004)
EVAB PCR	1 trypanosome/ml	*T. evansi* type B	(Njiru *et al.* 2006)
TvPRAC PCR	500 trypanosomes/ml	*T. vivax*	(Fikru *et al.* 2014)

3. Materials and methods

Study areas

The study was conducted in selected districts (weredas) of Tigray and pastoral areas of Afar, representing tsetse-free areas of Ethiopia. Tigray region is located in the northern part of Ethiopia between longitudes 36°27' E and 39°59' E and latitudes 12°15' N and 14°57' N (Figure 3.1). It shares international boundaries with Eritrea and Sudan and regional boundaries with Amhara and Afar regions of Ethiopia. Tigray is divided into four zones and 35 weredas (Tassew 2000). Selected "tabias" or peasant associations from the districts of Raya-Azebo (southern zone), Tselemti (northwestern zone) and Kafta-Humera and Tsegede (western zone), were included. Afar region, one of the four major pastoral regions in Ethiopia, occupies an area of about 270,000 km^2 and is situated between longitudes 39°34' E and 42°28' E and latitudes 8°49' N and 14°30' N (CSA 2011). The region shares international boundaries with the State of Eritrea and Djibouti, as well as regional boundaries with the regions of Tigray, Amhara, Oromia and Somali (Figure 3.1). The Afar region consists of 5 administrative zones (sub-regions) (David & Thomas 2013). Taking into account the accessibility to the pastoral communities, "kebeles" or sampling stations were selected in the districts of Megale (zone 2), Awash Fentale and Amibara (zone 3) and Gulina and Yalo (zone 4).

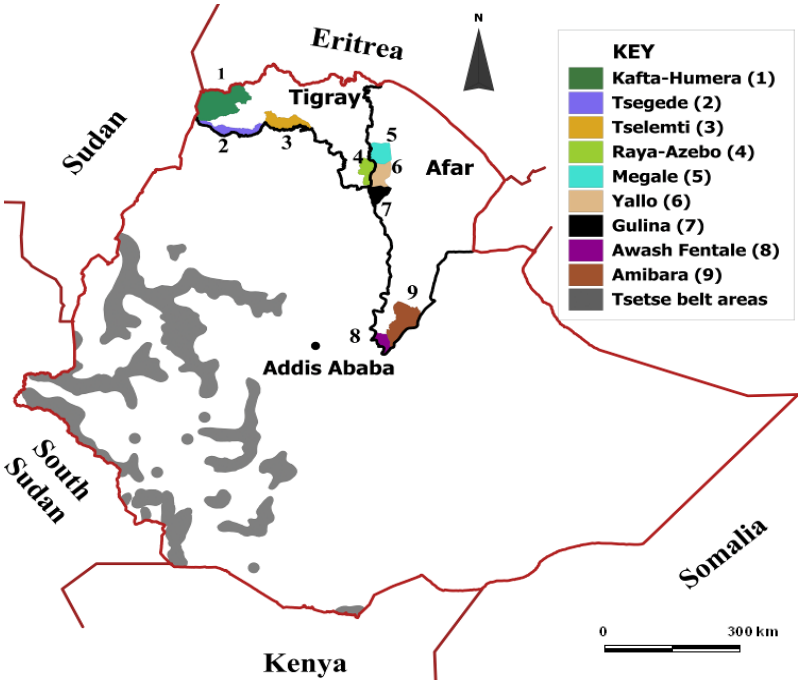

Figure 3.1: Map of Ethiopia showing study districts in Tigray and Afar regions and tsetse belt areas.

Ethics statement

The Animal Experimentation Ethics Committee (AEEC) of the Institute of Tropical Medicine, Antwerp, Belgium (ITM) advised on the protocol for collection of blood samples from dromedary camels, cattle, equines and small ruminants in Ethiopia (EXT2012-1). The standard ethical guidelines were also in line with the national guidelines of the Ethiopian Ministry of Livestock and Fishery Development and the Institutional Review Board of the Ministry of Science and Technology.

Study design, study animals and specimen collection

Considering 95% confidence level and average prevalence of 30% (Fikru *et al.* 2012), the number of specimens to collect was planned according to Thrusfield (2007) as 323= $(1.96)^2$ x Pexp(1-Pexp)/d^2; where: n = required sample size, d= absolute precision required (d=0.05), Pexp = expected prevalence of the disease. A cross sectional study was conducted from February till July 2013 on 1811 domestic animals comprising 754 dromedary camels, 493 cattle, 264 goats, 181 sheep, 84 donkeys, 25 horses and 10 mules. The animals were sampled at watering and grazing points and at veterinary clinics where they were brought for accaricide spraying and

vaccination. Individual study subjects from willing owners were randomly selected regardless of age, gender and body condition (Thrusfield 2007). From each animal, 9 ml of jugular vein blood was collected in a heparinised Venosafe tube (Terumo, Leuven, Belgium), labelled with a unique code, placed in a coolbox at 4°C and processed as described below.

Packed cell volume (PCV) and microhaematocrit centrifugation technique (mHCT)

The microhaematocrit (mHCT) was performed as described by (Woo 1970). Briefly, four microhaematocrit capillary tubes were filled with approximately 50 µl of blood from the Venosafe tube and stoppered with sealant. After centrifugation at 11,000 g for 5 min, the PCV was recorded and the tubes were mounted in a specially designed viewing chamber and examined under the microscope at 10x16 magnification for the presence of motile trypanosomes at the level of the buffy coat as described by Fikru *et al*. (2012). Animals with confirmed presence of trypanosomes were treated free of charge with 0.25 mg/kg melarsamine hydrochloride (cymelarsan) in the case of camels or with 0.5 mg/kg isometamidium chloride (samorin, trypamidium) or 7 mg/kg diminazene aceturate (berenil) in the case of ruminants.

Preparation of plasma and buffy coat specimens

The blood collected in the heparinised Venosafe tubes was centrifuged for 10 min at 1,500 g and plasma was collected with a single use plastic transfer pipette into 2 ml tubes with screwcaps (Sarstedt, Nümbrecht, Germany). Plasma was stored at 4 °C until testing for specific antibodies with CATT/*T. evansi* and subsequently frozen at -20 °C. From the remaining blood specimen, 500 µL of buffy coat layer were collected by means of a micropipette with a filter tip and mixed with an equal volume of guanidium EDTA buffer (GEB; 6M guanidium chloride, 0.2 M EDTA, pH 8.0) and stored at ambient temperature until DNA extraction (De Winne *et al.* 2014). Of those animals that were parasitologically positive, part of the buffy coat was collected for cryopreservation in liquid nitrogen for later isolation of the parasite according to Pyana et al. 2011 (Pyana *et al.* 2011).

CATT/*T. evansi*

Detection of *T. evansi* specific antibodies was carried out by CATT/*T. evansi* on plasma that was prediluted 1:4 in CATT diluent, according to the instructions of the manufacturer (Institute of Tropical Medicine, Antwerp, Belgium).

Immune trypanolysis test for surra

From each plasma specimen, 30 µl were spotted on Whatman 4 filter paper (Whatman, Maidstone, UK) in Ethiopia and shipped to the Institute of Tropical Medicine, Antwerp, Belgium. For elution of plasma and test procedures, the protocol developed by Camara and co-workers, with modifications, was employed (Camara *et al.* 2014). Briefly, from each filter paper, two 6 mm

diameter disks were punched and placed in a well of a flat bottom microlon microtitre plate (Greiner Bio-One, Wemmel, Belgium). Antibodies were eluted overnight at 4 °C in 40 µl of fetal bovine serum (FBS) followed by 1 hour on a plate shaker at ambient temperature. Twenty µl of the eluted fraction were transferred into a well of a U-bottom polystyrene microtitre plate (Sterilin, Newport, UK). Next, *T. evansi* RoTat 1.2 trypanosomes, grown in a mouse, were diluted in ice-cold guinea pig serum (GPS) and kept on ice to obtain a suspension of 5 trypanosomes per microscopic field according to the matching method (Herbert & Lumsden 1976). Twenty µl of this suspension were added to each well of the microtiter plate with the eluted specimens and incubated at ambient temperature for 1 hour. Antibody mediated complement lysis was assessed by dispensing 5 µl of the reaction mixture on a microscope slide, covered by a 18 x 18 mm cover slip and examination at 25 x 10 magnification under a phase-contrast microscope. Trypanolysis was considered positive when at least 50% of the trypanosomes were lysed (Camara *et al.* 2014).

DNA extraction

DNA extraction was performed with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). Since unexpected clotting of the buffy coat specimens preserved in GEB was observed, 200 µl of tissue lysis buffer and 50 µl of proteinase K (Roche Diagnostics, Mannheim, Germany) were added to the 1 ml buffy coat-GEB mixture followed by digestion for 90 min at 56 °C under constant shaking at 1,000 rpm. Eventually, DNA was extracted from 240 µl of this mixture and stored at -20 °C until use. DNA concentrations were measured in the Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA) or the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, USA).

PCR

All PCR amplifications were carried out in 200 µl thin-wall PCR tubes (ABgene, Epsom, UK) in a T3 thermocycler 48 (Biometra, Göttingen, Germany). Amplified products were visualised under UV after electrophoresis in a 2% agarose gel at 135 V for 30 minutes and staining with ethidium bromide. To check the quality of DNA, a PCR targeting vertebrate cytochrome b was performed (Aref *et al.* 2013; Kocher *et al.* 1989). To detect *T. evansi* type A, the RoTat 1.2 PCR was conducted (Claes *et al.* 2004) while the EVAB PCR was used for the detection of *T. evansi* type B (Njiru *et al.* 2006). Detection of *T. vivax* was performed by means of *Tv*PRAC PCR (Fikru *et al.* 2014). ITS1-PCR was used to test part of the specimen collection for *T. congolense*, *T. theileri* and *Trypanozoon* (Desquesnes *et al.* 2001). Each PCR assay was done in 25 µl reaction volumes with 12.5 µl HotStar Taq polymerase master mix (Qiagen,Leipzig, Germany) containing 2.5 units HotStar Taq DNA polymerase, 1 x PCR buffer with 1.5 mM $MgCl_2$ and 200 µM of each dNTPs, 0.8 µM of each primer (Biolegio, Amsterdam, Netherlands), 8 µl accugene water (Lonza, Verviers, Belgium) and 2.5 µl of template DNA.

The target genes, primer names and sequences and expected amplicon lengths are represented in Table 3.2. Compared to the references cited in the table, some minor changes

were made at the level of the polymerase and master mix, the initial denaturation step and the numbers of cycles. Cycling conditions for the different PCRs were as follows. Cytochrome B PCR: 94 °C for 15 min and 35 cycles of 30 sec at 94 °C, 30 sec at 52 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C. RoTat 1.2 PCR: 94 °C for 15 min and 40 cycles of 30 sec at 94 °C, 30 sec at 59 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C. EVAB PCR: 94 °C for 15 min and 30 cycles of 30 sec at 94 °C, 30 sec at 60 °C, 60 sec at 72 °C and final extension for 1 min at 72 °C. *Tv*PRAC PCR: 94 °C for 15 min and 30 cycles of 30 sec at 94 °C, 30 sec at 63 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C. ITS-1 PCR: 94 °C for 15 min and 40 cycles of 30 sec at 94 °C, 30 sec at 60 °C, 30 sec 72 °C and final extension for 5 min at 72 °C.

Table 3.2: Specifications of the PCR assays used in the study.

Taxon	Target gene	Primers	Primer sequences	Amplicon length	Reference
Vertebrates	Cytochrome b	L14841 H15149	5'-CCATCCAACATCTCAGCATGATGAAA-3' 5'-GCCCCTCAGAATGATATTTGTCCTCA-3'	400 bp	Adapted from (Kocher *et al.* 1989)
T. evansi Type A	VSG RoTat 1.2	RoTat1.2-F RoTat1.2-R	5'-GCGGGGTGTTTAAAGCAATA-3' 5'-ATTAGTGCTGCGTGTGTTCG-3'	205 bp	Adapted from (Claes *et al.* 2004)
T. evansi Type B	Minicircle	EVAB-1 EVAB-2	5'-ACAGTCCGAGAGATAGAG-3' 5'-CTGTACTCTACATCTACCTC-3'	436 bp	Adapted from (Njiru *et al.* 2006)
T. vivax	Proline racemase	*Tv*PRAC-F *Tv*PRAC-R	5' CGCAAGTGGACCGTTCGCCT- 3' 5' ACGCGGGGCGAACAGAAGTG-3'	239 bp	Adapted from (Fikru *et al.* 2014)
Diverse *Trypanosoma* species	ITS-1	ITS-1 F ITS-1 R	5'-TGTAGGTGAACCTGCAGCTGGATC-3' 5'-CCAAGTCATCCATCGCGACACGTT- 3'	*T. vivax* 150 bp, *T. theileri* 350 bp, *Trypanozoon* 450 bp, T. *congolense* 650 bp	(Fikru *et al.* 2012)

Data analysis

All data were recorded in Microsoft Excel. STATA /MP 13.1 (StataCorp. 2013) was used for statistical analysis. Pearson's chi- square was used to check the distribution of the sample sizes of the different host species within the two regions. Percentages with 95% confidence interval (CI) were used to express prevalence. Logistic regression (using a penalised log likelihood if necessary) was applied for assessing differences in prevalence of AAT, in function of the diagnostic tests, between domestic animal species (except in equines). Linear regression was used to evaluate the effect of infection (test positive) on PCV values (dependent variable). To assess agreement between the diagnostic tests, Cohen's kappa coefficient was calculated and interpreted according to (Landis & Koch 1977). The Z test (Kappa/standard error) was used to

verify whether Kappa values were significant or not. P-values < 0.05 were considered as significant.

4. Results

In total, 1811 animals were sampled of which 959 (53%) in Tigray and 852 (47%) in Afar. In general, there was statistically significant interaction (X^2 = 330.12, p<0.001) between regions and sampled domestic animal species, i.e. more cattle and camels were sampled in Tigray than in Afar, while more sheep and goats were sampled in Afar than in Tigray.

Parasite detection

In 68 animals, trypanosomes were detected (Table 3.3). Thus, the overall parasitologically confirmed prevalence of trypanosomosis was 3.8% (CI 2.9-4.6%) with 4.7% (CI 3.4-6.0%) in Tigray and 2.7% (CI 1.6-3.8%) in Afar. No trypanosomes were detected in equines. The parasitological prevalence in cattle (7.3%, CI 5.0-9.5%) was significantly higher (z=2.53, p=0.011) than in camels (4.0%, CI 2.6-5.4%), sheep (0.6%, CI 0-1.7%) and goats (0.4%, CI 0-1.2%).

Serology

With CATT/*T. evansi*, antibodies were detected in 354 animals (Table 3.3). Thus, the overall seroprevalence was 19.6% (CI 17.7-21.4%) with 24.6% (CI 21.9-27.3%) in Tigray and 13.9% (CI 11.5-16.2%) in Afar. Among the equines, CATT/*T. evansi* was only positive in donkeys (10.7%, CI 4.0-17.4%). The overall seroprevalence was significantly higher (z=9.39, p<0.001) in cattle (37.3%, CI 33.1-41.6%) than in camels (13.7%, CI 11.2-16.1%), in goats (13.3%, CI 9.2-17.4%), in sheep (12.7%, CI 7.8-17.6%) and in donkeys (10.7%, CI 4.1-17.4%). With the TL (Table 3.3), *T. evansi*-specific antibodies were detected only in 34 animals (30 camels, 2 goats, 1 sheep and 1 donkey). Thus, the seroprevalence in TL was 1.9 % (34/1811, CI 1.3-2.5%). Kappa statistics indicated a poor but significant agreement between CATT/*T. evansi* and TL (p < 0.001, Table 3.4).

Molecular diagnosis

The overall molecular prevalence of *T. evansi* type A assessed with RoTat 1.2 PCR was 145/1811 or 8.0% (CI 6.8-9.3%) with 8.0% (CI 6.3-9.8%) in Tigray and 8.0% (CI 6.2-9.8%) in Afar (Table 3.3). The molecular prevalence of *T. evansi* type A in camels (11.7 %, CI 9.4-14.0%) was significantly higher (p<0.001) than in cattle (6.1%, CI 4.0-8.2%), donkeys (6.0%, CI 0.9-11.0%), goats (3.8%, CI 1.5-6.1%), and sheep (2.2%, 0.1-4.4%). The molecular prevalence of *T. evansi* type A was 28.0% (CI 10.4-45.6%) in horse and 10.0 % (CI 7.6-27.6%) in mule. Kappa statistics indicated a poor but significant agreement between RoTat 1.2 PCR and the antibody detection tests, TL and CATT/*T. evansi* (p < 0.001, Table 3.4). Among the 145 RoTat 1.2 PCR positives, only 71 were positive in CATT/*T. evansi* and only 18 were positive in TL. Four camels, all from Awash Fentale district in Afar, were found positive in EVAB PCR indicating the presence of *T. evansi* type B. All

four were negative in CATT/*T. evansi* and TL although one of them was also positive in RoTat 1.2 PCR suggesting a mixed infection.

The overall molecular prevalence of *T. vivax* assessed with *Tv*PRAC PCR was 54/1811 or 3.0% (CI 2.2-3.8%) with 3.3% (CI 2.2-4.5%) in Tigray and 2.6% (CI 1.5-3.7%) in Afar (Table 3.3). The molecular prevalences of *T. vivax* were 3.5% (CI 2.2-4.8%) in camels, 3.0% in goats (CI 1.0-5.1%), 2.6% (CI 1.2-4.1%) in cattle and 2.2% (CI 0.1-4.4%) in sheep and were not significantly different (p=0.925). All horses and mules were negative in *Tv*PRAC PCR. The molecular prevalence of *T. vivax* in cattle from Tigray was 3.2% (13/411) but was 0% in Afar. Among the 54 *Tv*PRAC PCR positives, 10 were also positive in CATT/*T. evansi* but were negative in RoTat 1.2 PCR. Only two camels and one goat were positive in both *Tv*PRAC PCR and RoTat 1.2 PCR.

Among the 68 parasitologically positive animals, 32 cattle, 1 camel and 1 sheep were negative in the RoTat 1.2 PCR, EVAB PCR and TvPRAC PCR. To check for the possibility that mHCT detected *T. theileri* and *T. congolense*, ITS1-PCR was run on their specimens. Four cattle were positive for *T. vivax* and two cattle specimens were positive for *T. theileri*. Ten were negative. No single one was positive for *T. congolense*. Eighteen cattle specimens showed a profile with amplicons of different lengths that could not be interpreted unequivocally. Among the CATT/*T. evansi* positive animals, 269 (77%) were negative in all PCR tests (165 cattle, 42 camels, 33 goats,22 sheep and 7 donkeys).

Packed cell volume (PCV)

In Table 3.5, the average PCV values and standard deviations (SD) are given according to the status of the animals in the mHCT, CATT/*T. evansi*, RoTat 1.2 PCR and *Tv*PRAC PCR. Camels that were found positive in those tests had a significantly lower average PCV than the animals that were negative in the different tests. The average PCV in TL positive camels (24.2% ± 3.4%) was not significantly different from TL negatives (25.7% ± 3.59%) (p=0.05). In cattle and equines, the average PCV value was significantly lower only in CATT/*T. evansi* positive animals. In sheep and goats, no significant differences in average PCV were observed.

Table 3.3: Test positives over total number of animals for each host species within each region.

Diagnostic test	Region	Host species						
		Cattle	**Camel**	**Goat**	**Sheep**	**Mule**	**Horse**	**Donkey**
mHCT	Tigray	32/411	11/343	1/60	1/64	0/10	0/25	0/46
	Afar	4/82	19/411	0/204	0/117	-	-	0/38
	Total	36/493	30/754	1/264	1/181	0/10	0/25	0/84
CATT/*T. evansi*	Tigray	169/411	39/343	12/60	14/64	0/10	0/25	2/46
	Afar	15/82	64/411	23/204	9/117	-	-	7/38
	Total	184/493	103/754	35/264	23/181	0/10	0/25	9/84
TL	Tigray	0/411	21/343	1/60	1/64	0/10	0/25	0/46
	Afar	0/82	9/411	1/204	0/117	-	-	1/38
	Total	0/493	30/754	2/264	1/181	0/10	0/25	1/84
RoTat 1.2 PCR	Tigray	23/411	33/343	6/60	4/64	1/10	7/25	3/46
	Afar	7/82	55/411	4/204	0/117	-	-	2/38
	Total	30/493	88/754	10/264	4/181	1/10	7/25	5/84
EVAB PCR	Tigray	0/411	0/343	0/60	0/64	0/10	0/25	0/46
	Afar	0/82	4/411	0/204	0/117	-	-	0/38
	Total	0/493	4/754	0/264	0/181	0/10	0/25	0/84
*Tv*PRAC PCR	Tigray	13/411	16/343	2/60	1/64	0/10	0/25	0/46
	Afar	0/82	10/411	3/204	3/117	-	-	3/38
	Total	13/493	26/754	8/264	4/181	0/10	0/25	3/84

Table 3.4: Degree of agreement between diagnostic tests.

Cross test	Observed (%)	Expected by chance (%)	Kappa	Z	p
CATT/*T. evansi* and ITL	81.45	79.31	0.10	8.45	<0.001
CATT/*T. evansi* and RoTat 1.2 PCR	80.29	75.58	0.19	9.31	<0.001
RoTat 1.2 PCR and ITL	92.10	90.42	0.176	9.75	<0.001

Table 3.5: Average PCV of the animals according to their status in the different diagnostic tests.

Test	Species	% PCV test negative ± SD[a]	% PCV test positive ± SD[a]	Regression coefficient value	t[b]	P[c]
mHCT	Camels	25.8 ± 3.53	21.5 ± 2.53	-4.23	-6.50	< 0.001*
	Cattle	25.9 ± 5.25	24.9 ± 5.49	-0.97	-1.07	0.287
CATT/*T. evansi*	Camels	25.9 ± 3.46	23.8 ± 3.87	-2.09	-5.59	< 0.001*
	Cattle	26.6 ± 5.69	24.6 ± 4.19	-2.02	-4.20	< 0.001*
	Equines	33.6 ± 6.3	27.9 ± 7.9	-5.71	-2.56	0.012*
	Goats	26.7± 5.84	24.9 ± 4.57	-1.79	-1.73	0.084
	Sheep	25.1 ± 5.57	22.9 ± 6.11	-2.12	-1.77	0.088
RoTat	Camels	25.0 ± 3.49	23.7 ± 3.81	-2.16	-5.39	< 0.001*
1.2 PCR	Cattle	25.8 ± 5.25	26.3 ± 5.56	0.53	0.54	0.591
	Equines	33.2 ± 6.6	33.1 ± 7.1	-0.98	-0.05	0.960
	Goats	26.5 ± 5.68	23.3 ± 5.89	-3.29	-1.79	0.074
	Sheep	24.8 ± 5.71	25.5 ± 3.89	0.74	0.26	0.796
*Tv*PRAC	Camels	25.7 ± 3.57	23.8 ± 3.77	-1.89	-2.65	0.008*
	Cattle	25.9 ± 5.30	23.1 ± 2.91	-2.83	-1.92	0.056
	Equines	33.2 ± 6.6	33.2 ± 5	-0.003	0.00	0.999
	Goats	26.4 ± 5.71	26.9 ± 6.30	0.543	0.26	0.792
	Sheep	24.8 ± 5.70	23.8 ± 4.33	-1.05	-0.36	0.716

[a] SD: standard deviation; [b] t: Student's t distribution value; [c] P: probability; * statistically significant reduction in PCV

5. Discussion

In this cross sectional study, the mHCT, CATT/*T. evansi*, RoTat 1.2 TL and RoTat 1.2 PCR, EVAB PCR and *Tv*PRAC PCR were used to assess the non-tsetse transmitted AAT prevalence in domestic animals in two regions of northern Ethiopia, Tigray and Afar. The overall prevalence of AAT as assessed with mHCT was 3.75% which was similar to AAT prevalence reported in cattle from other tsetse-free areas in Ethiopia (3.2% in Gondar and Bale Lowlands) using the same technique (Fikru *et al.* 2012). This is probably underestimating the real prevalence since mHCT is acknowledged to detect <50% of infections with low parasitaemia (Monzón *et al.* 1990; Murray *et al.* 1977). Although only one goat and one sheep were positive in mHCT, this finding confirms the presence of trypanosomosis in small ruminants (Sinshaw *et al.* 2006; Samson & Frehiwot 2010; Tadesse & Tsegaye 2010; Kebede *et al.* 2009). The fact that no single equine was positive in mHCT while some of them were positive in the *T. evansi* specific RoTat 1.2 PCR and the *T. vivax* specific *Tv*PRAC PCR, indicates that in these animals the parasitaemia level remained under the lower detection limit of mHCT (about 60 trypanosomes/ml), (OIE 2013b).

With RoTat 1.2 PCR, it was confirmed that all domestic animals are susceptible to infection with *T. evansi* type A but that camels and horses are particularly at risk (Desquesnes *et al.* 2013b; Desquesnes *et al.* 2013a). With EVAB PCR, we report for the first time the presence of *T. evansi* type B in camels in Ethiopia. To date, *T. evansi* type B has only been isolated from camels in Kenya although indirect evidence exists that it also circulates in Sudan (Salim *et al.* 2011; Boid 1988; Borst *et al.* 1987; Ngaira *et al.* 2005). Furthermore, Hagos et al. suggested the existence of non-RoTat 1.2 *T. evansi* in camels from Bale zone in Ethiopia based on their finding that about one third of parasitologically positive camels were negative in CATT/*T. evansi* (Hagos *et al.* 2009). Also in our study, all four camels with *T. evansi* type B were negative in CATT/*T. evansi*. These data suggest that *T. evansi* type B is not confined to Kenya but may occur in more East African countries and even beyond, thus calling for the adaptation of serological and molecular diagnostic tests, like CATT/*T. evansi* and RoTat 1.2 PCR, to ensure detection of both types of *T. evansi* without compromising specificity.

Our study also confirms that *T. vivax* can infect diverse domestic animal species, including donkeys (Hoare 1972). The overall molecular prevalence of *T. vivax* as assessed with *Tv*PRAC PCR was lower than reported in other studies (Fikru *et al.* 2012; Fikru *et al.* 2014). The present study shows that camels in Ethiopia can be infected with *T. vivax* and that infection is associated with morbidity reflected by a significant reduction in PCV. Co-infections with *T. vivax* and *T. evansi* were rare (2 camels, 1 goat) but characterised by low PCV (20-22.5%). Mixed infection by both parasites was also reported in (Takeet *et al.* 2013).

As expected, ITS1 PCR confirmed the absence of *T. congolense* in the mHCT positive animals that were negative in RoTat 1.2 PCR and *Tv*PRAC PCR but revealed four *T. vivax* infections that were not picked up by *Tv*PRAC PCR. Interestingly, ten mHCT positive animals remained negative in all PCRs. In the single sheep, the presence of the non-pathogenic *T. melophagium* cannot be ruled out but the other nine negatives remain unexplained (Gibson *et al.* 2010; Büscher & Friedhoff 1984). Also unexplained remain the 18 cattle specimens showing a complex amplicon profile in ITS1 PCR, including a putative *T. vivax* specific 150 bp amplicon (see figure 3.2 for an example). In a previous study, which led to the development of the *Tv*PRAC PCR, we observed that the ITS1 PCR can generate non-specific amplicons in the presence of cattle DNA rendering unequivocal interpretation of the results impossible (Fikru *et al.* 2012). Given the complexity of the profile, we didn't undertake sequencing of the undefined amplicons. Although the analytical sensitivity of *Tv*PRAC is lower than of ITS1 PCR, it is still higher than of mHCT (Fikru *et al.* 2014). Therefore, mHCT positive and *Tv*PRAC negative but ITS1 *T. vivax* positive specimens may be due to particular *T. vivax* strains not detectable in *Tv*PRAC. To further investigate these unexplained results, it will be necessary to isolate the trypanosomes detected in the mHCT, which will be particularly challenging in case of *T. vivax*. Indeed, *T. vivax* is notoriously difficult to grow in laboratory rodents and/or in culture (Gathuo *et al.* 1987; D'Archivio *et al.* 2011).

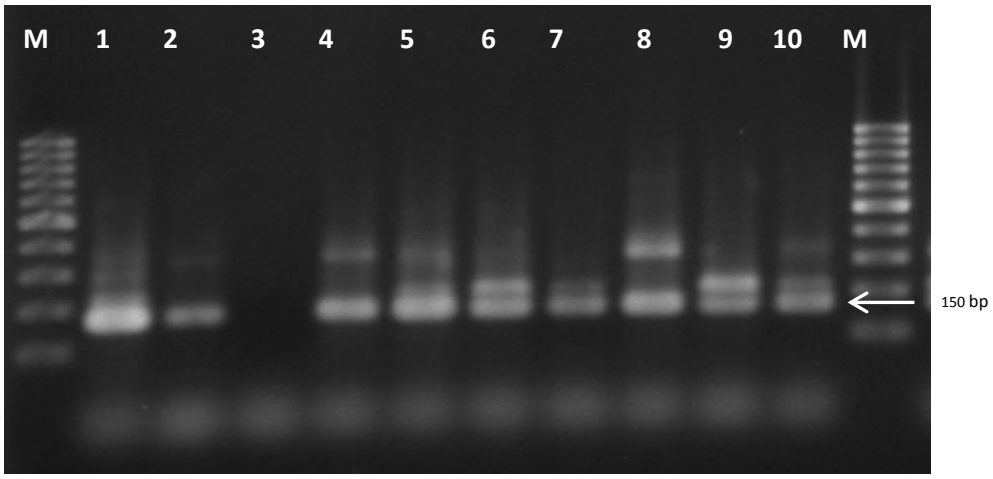

Figure 3.2. Agarose gel showing non-specific ITS1 PCR amplicons on mHCT positive buffy coat samples of cattle (lanes 1-10). M= 100 bp marker; the *T. vivax* specific amplicon is 150 bp long.

Seroprevalence, as assessed with CATT/*T. evansi* was much higher than molecular prevalence which is not unexpected for several reasons. First, CATT/*T. evansi* cannot distinguish current from cured infection as detectable level of antibodies can persist for 2.3–22.6 month after trypanocidal treatment (Hilali *et al.* 2004; Monzón *et al.* 2003). Secondly, in particular in chronic infections, parasitaemia can be well below the detection limit of parasitological and even molecular diagnostic tests, a phenomenon well known in human African trypanosomosis but less studied in AAT (Büscher 2014; Deborggraeve & Büscher 2012). Finally, as CATT/*T. evansi* is not 100% specific, false positive cases do occur (Verloo *et al.* 1998).

Still, the poor agreement between CATT/*T. evansi* and TL is puzzling. Both serological tests detect antibodies against the VSG RoTat 1.2 that is considered specific for *T. evansi* type A. Although a limited loss in sensitivity of TL when performed on filter paper eluates may be expected other factors may cause this discrepancy (Camara *et al.* 2014; Holland *et al.* 2002). While TL detects exclusively variant specific antibodies, CATT/*T. evansi* detects also antibodies directed against non-variant specific epitopes of VSG RoTAt 1.2 and other surface exposed antigens. Thus, infection with other trypanosomes, e.g. *T. vivax*, may lead to a positive result in CATT/*T. evansi* as was suggested in a study on bovine trypanosomosis in Suriname (Van Vlaenderen 1996; Uzcanga *et al.* 2004; Büscher 2001). This cross-reactivity caused by *T. vivax* infection may explain why all CATT/*T. evansi* positive cattle specimens remained negative in TL. However, it provides no explanation why the 30 cattle specimens that were positive in RoTat 1.2 PCR remained negative in TL and why from the 145 RoTat 1.2 PCR positives, only 71 were also positive in CATT/*T. evansi*. Is it possible that the target sequence of RoTat 1.2 PCR is also present

in some particular *T. vivax* strains circulating in Afar and Tigray but that the gene containing that sequence is a pseudogene or a gene that is not expressed during an infection? As we were not able to isolate *T. vivax* strains during this study, a conclusive answer to this question cannot be given.

If one considers a low PCV as a morbidity marker, it is striking that mainly camels are susceptible to AAT as disease. Indeed, camels that were positive in mHCT, CATT/*T. evansi*, RoTat 1.2 PCR and TvPRAC PCR had a significantly lower PCV than camels that were negative in all these tests. Among the other host species, only cattle and equines that were positive in CATT/*T. evansi* had a significantly lower PCV than CATT/*T. evansi* negative animals again suggesting that most CATT/*T. evansi* positive animals were actually infected, whether with *T. evansi* or *T. vivax*.

Among the parasitologically positive animals, three quarter presented without or with only mild symptoms. As in the study region, it is common to treat only sick camels and bovines with trypanocidal drugs such as diminazine and isometamidum, asymptomatic infections remain without treatment and constitute an uncontrolled reservoir for the disease.

Our study has some limitations. Although intended, it was not possible to compare the AAT prevalence between Tigray and Afar since the number of examined individuals per animal species was considerably different between two study regions. Also, the number of examined small ruminants and equines was below the intended number of 323. In small ruminants, we observed 13% seroprevalences and 2-4% molecular prevalences. Hence, the 264 goats and 181 sheep that were examined are sufficiently high to obtain statistically significant prevalence data. Finally, no stained blood preparations were prepared that would have allowed morphological characterisation of those parasites that were detected in the mHCT but that remained negative in the species-specific PCRs.

In the next chapter, we will describe how we isolated and characterized *T. evansi* stocks from the cryopreserved blood of animals that were found parasitologically positive in this epidemiological survey.

New *Trypanosoma evansi* type B isolates from Ethiopian dromedary camels

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Author contributions: HB, NVR and PB conceived and designed the experiments and analysed the data. HB performed strain isolation. NVR performed *in vitro* adaptation and drug sensitivity experiments. HB, NVR, PB, BMG and TG contributed to reagents, materials, analysis tools. HB and NVR wrote the manuscript. All authors revised and approved the final manuscript.

1. Abstract

Trypanosoma (T.) evansi is a dyskinetoplastic variant of *T. brucei* that has gained the ability to be transmitted by all sorts of biting flies. *T. evansi* can be divided into type A, which is the most abundant and found in Africa, Asia and Latin America and type B, which has so far been isolated only from Kenyan dromedary camels. This study aimed at the isolation and the genetic and phenotypic characterisation of type A and B *T. evansi* stocks from camels in Northern Ethiopia.

T. evansi was isolated in mice by inoculation with the cryopreserved buffy coat of parasitologically confirmed animals. Fourteen stocks were thus isolated and subject to genotyping with PCRs targeting type-specific variant surface glycoprotein genes, mitochondrial minicircles and maxicircles, minisatellite markers and the F1-ATP synthase γ subunit gene. Nine stocks corresponded to type A, two stocks were type B and three stocks represented mixed infections between A and B, but not hybrids. One *T. evansi* type A stock was completely akinetoplastic. Five stocks were adapted to *in vitro* culture and subjected to a drug sensitivity assay with melarsomine dihydrochloride, diminazene diaceturate, isometamidium chloride and suramin. *In vitro* adaptation induced some loss of kinetoplasts within 60 days. No correlation between drug sensitivity and absence of the kinetoplast was observed. Sequencing the full coding sequence of the F1-ATP synthase γ subunit revealed new type-specific single nucleotide polymorphisms and deletions.

This study addresses some limitations of current molecular markers for *T. evansi* genotyping. Polymorphism within the F1-ATP synthase γ subunit gene may provide new markers to identify the *T. evansi* type that do not rely on variant surface glycoprotein genes or kinetoplast DNA.

2. Introduction

Trypanosomes are characterised by the presence of a structure called kinetoplast that corresponds with the DNA (kDNA) of their unique mitochondrion. *T. brucei* kDNA contains 20-50 copies of maxicircles (about 23 kb) and a highly diverse set of thousands of minicircles (about 1 kb). Maxicircles contain rRNA coding regions and genes coding for subunits of the respiratory chain complexes while minicircles code for guide RNAs required for editing (Schnaufer *et al.* 2002).

T. equiperdum and *T. evansi* are dyskinetoplastic ($kDNA^-$) since they lack part of the kDNA (Claes *et al.* 2005; Lai *et al.* 2008; Schnaufer *et al.* 2002; Carnes *et al.* 2015). *T. equiperdum* typically has retained maxicircles, in some cases with substantial deletions, but has lost its minicircle diversity. *T. evansi* does not have maxicircles and either shows minicircle homogeneity or are akinetoplastic (kDNA°) (Ou *et al.* 1991; Lun & Vickerman 1991; Ventura *et al.* 2000; Schnaufer *et al.* 2002). Based on their minicircle restriction digestion profile, *T. evansi* can be divided into type A and type B (Njiru *et al.* 2006; Borst *et al.* 1987).

T. evansi type A is the most abundant and is found in Africa, South America and Asia. It is characterised by the presence of the gene for the variant surface glycoprotein (VSG) RoTat 1.2. This RoTat 1.2 VSG is expressed early during infections resulting in the detectability of anti-RoTat 1.2 antibodies in animals infected with *T. evansi* type A (Verloo *et al.* 2001; Bajyana Songa & Hamers 1988). In contrast, *T. evansi* type B is far less common and has so far been isolated only from camels in Kenya (Borst *et al.* 1987; Ngaira *et al.* 2005). More recently, serological and molecular evidence for the presence of *T. evansi* type B in Sudan, Ethiopia and Chad was published (Birhanu *et al.* 2015a; Hagos *et al.* 2009; Salim *et al.* 2011; Boid 1988; Sánchez *et al.* 2015). *T. evansi* type B lacks the RoTat 1.2 gene and as a consequence, infections with this type are not detected with serological and molecular tests based on RoTat 1.2 VSG, such as the CATT/*T. evansi* and RoTat 1.2 PCR (Njiru *et al.* 2006; Bajyana Songa & Hamers 1988; Claes *et al.* 2004; Ngaira *et al.* 2005). So far, three molecular tests have been developed for the identification of *T. evansi* type B: the EVAB PCR, targeting a type B-specific minicircle DNA sequence, and a PCR and a LAMP targeting a type B-specific VSG JN 2118Hu (Njiru *et al.* 2006; Ngaira *et al.* 2005; Njiru *et al.* 2010). *T. equiperdum* is the least known parasite of the *Trypanozoon* group, with very few isolates available for research, albeit new stocks were isolated from Ethiopian and Venezuelan horses recently (Hagos *et al.* 2010c; Sánchez *et al.* 2015).

Unlike *T. brucei*, *T. evansi* and *T. equiperdum* cannot develop in tsetse flies due to their inability to transform into the procyclic life stage. They can only survive in a mammalian host where they produce ATP exclusively through glycolysis. In contrast to bloodstream forms, ATP production in procyclic trypanosomes relies on oxidative phosphorylation and, therefore, on the capacity to express the full set of corresponding mitochondrial genes, including some which are encoded by the kDNA (Dean *et al.* 2013; Schnaufer *et al.* 2002). Bloodstream forms of *T. evansi,*

T. equiperdum and laboratory-generated *T. brucei* strains that have lost all or critical parts of their kDNA, can survive without kDNA due to specific single amino acid mutations in the gamma (γ) subunit of the mitochondrial F1-ATP synthase (Dean *et al.* 2013). Interestingly, the specific mutations/deletions in the C-terminal region of F1-ATP γ subunit enable differentiation among the *Trypanozoon* strains (Lai *et al.* 2008). Furthermore, when the F1-ATP γ subunits of *T. evansi* type A (A281del), *T. equiperdum* (A273P) and the laboratory-generated *T. brucei* (L262P) strains are overexpressed in a *T. brucei* γ subunit knock out strain, the latter can survive after loss of its kinetoplast after treatment with DNA intercalating drugs such as acriflavin or ethidium bromide (Schnaufer 2010; Dean *et al.* 2013). Once the genetically modified *T. brucei* are independent from kDNA maintenance and expression, they become multidrug resistant to the diamidine and phenanthridine class of drugs (Gould & Schnaufer 2014).

In *T. evansi,* drug resistance has been reported in several type A strains originating from Africa, Asia and Latin America (El Rayah *et al.* 1999; Payne *et al.* 1994a; Boid *et al.* 1989; Zhou *et al.* 2004). Some Chinese strains appear to be innately resistant to the phenanthridine class of drugs (Brun & Lun 1994). In contrast, nothing is known on the drug susceptibly of the *T. evansi* type B strains. In Chapter 3, we reported that *T. evansi* infections are very common in camels, equines, cattle and small ruminants in Tigray and Afar provinces in Northern Ethiopia (Birhanu *et al.* 2015a). We also provided molecular and serological evidence that both *T. evansi* type A and type B occur in these provinces. As described in Chapter 3, of those dromedary camels that were parasitologically positive, buffy coat samples were collected and cryopreserved in liquid nitrogen for later isolation of the parasite. We here report on the isolation, adaptation to *in vitro* culture, genetic and phenotypic characterisation and *in vitro* drug sensitivity of *T. evansi* type A and B from Northern Ethiopia.

3. Materials and methods

Ethics statement

The Animal Experimentation Ethics Committee (AEEC) of the Institute of Tropical Medicine (ITM) advised on the protocol for collection of blood samples from dromedary camels (EXT2012-1) and for the isolation of trypanosomes via inoculation of mice (EXT2012-2) at the College of Veterinary Medicine, Mekelle University. The study protocol for *in vivo* expansion of trypanosomes at ITM was approved by the AEEC (BM2013-1). Collecting blood from camels and experiments on mice were conducted according to the national guidelines of the Ethiopian Ministry of Livestock and Fishery Development and the Institutional Review Board of the Ministry of Science and Technology.

***In vivo* isolation of parasites from cryopreserved buffy coat in mice**

Details on the collection and cryopreservation of buffy coat samples from dromedary camels that were parasitologically confirmed in the micro haematocrit centrifugation technique have been fully described in Chapter 3. Two hundred µl of thawed buffy coat were inoculated

intraperitoneally (IP) in two 25–30 g Swiss albino mice that were immunosuppressed with 0.16 µg kg^{-1} body weight dexamethasone (Shanghai Central Pharmaceutical, China) one day prior to inoculation (Sultana 1996). Parasitaemia was checked in 5 µl of tail blood using the matching method (Herbert & Lumsden 1976), starting from day 7 post-infection and subsequently on every third day. As soon as trypanosomes were detected in at least one mouse, the animal was anaesthetised (the other kept as a backup), its blood was collected on heparin by heart puncture, diluted in an equal volume of phosphate buffered saline glucose (PSG; 7.5 g/l $Na_2HPO_42H_2O$, 0.34 g/l NaH_2 PO_4H_2O, 2.12 g/l NaCl, 10 g/l D-glucose, pH 8) and subinoculated into four naïve mice (200 µl each) which were monitored for parasitaemia as described above. Mice used as backup were euthanised when the newly infected mice became positive. When parasitemia reached about ± $10^{7.8}$ cells ml^{-1} of blood, two of these parasitaemic mice were euthanised (the other two were kept as back up) and blood was taken for subinoculation into four other naïve mice. This protocol was repeated until the parasitaemia reached about $10^{8.4}$ cells ml^{-1}. At this stage the stock was considered *in vivo* adapted. All four mice were anaesthetised and exsanguinated by heart puncture in an equal volume of Triladyl-egg yolk-phosphate buffered saline glucose (TEP) cryomedium (Pyana *et al.* 2011) for cryopreservation in 1 ml aliquots.

***In vivo* expansion and purification of parasite populations**

Cryostabilates were thawed in a water bath at 37 °C and diluted in PSG to 1 trypanosome per field (± $10^{5.7}$ cells ml^{-1}). Two-hundred µl volumes were injected IP in two naïve 20–30 g female OF-1 mice (Charles River, Belgium). Starting from three days post infection (DPI), parasitaemia was monitored daily and harvested at first peak parasitemia, typically at day 4 to 5 post-infection, as described above. Volumes of 0.5 ml of the blood were run over a mini Anion Exchange Centrifugation Technique (mAECT) column to separate the trypanosomes from the blood (Büscher *et al.* 2009). The trypanosomes eluted from the column were washed twice with 5 ml ice-cold PSG by centrifugation at 1500 g for 15 min. After the last centrifugation, the supernatant PSG was discarded and the trypanosome sediment was re-suspended in 100 µl of PSG. Part of this suspension was used for *in vitro* culture adaptation. The remainder was centrifuged at 1500 g for 5 min and the sediment was frozen at -80°C until DNA extraction. The isolates used for *in vivo* isolation and expansion and the corresponding *T. evansi* type A and B specific PCR result on their corresponding buffy coat DNA are indicated in Table 4.1.

Table 4.1: List of Ethiopian *T. evansi* isolates with data on origin and results in RoTat 1.2 PCR and EVAB PCR performed on DNA extracted from the buffy coat specimens from the infected camels. pos: positive, neg: negative.

Stabilate code	**Region**	**District**	**Station**	**RoTat 1.2 PCR**	**EVAB PCR**	***In vivo* subpassages before first cryostabilate**	***In vivo* expansion at ITM**
MCAM/ET/2013/MU/01	Afar	Megalle	Adahara	pos	neg	3	yes
MCAM/ET/2013/MU/02	Tigray	Raya-Azebo	Chercher	pos	neg	5	yes
MCAM/ET/2013/MU/03	Tigray	Raya-Azebo	Kukufto	pos	neg	5	no
MCAM/ET/2013/MU/04	Tigray	Raya-Azebo	Chercher	pos	neg	3	yes
MCAM/ET/2013/MU/05	Tigray	Raya-Azebo	Balla	pos	neg	4	yes
MCAM/ET/2013/MU/06	Tigray	Raya-Azebo	Balla	pos	neg	3	yes
MCAM/ET/2013/MU/07	Afar	Yallo	Gubidera	pos	neg	2	yes
MCAM/ET/2013/MU/08	Afar	Golina	Ullel-ella	pos	neg	2	yes
MCAM/ET/2013/MU/09	Tigray	Raya-Azebo	Kukufto	pos	neg	3	yes
MCAM/ET/2013/MU/10	Afar	Awash Fentale	Alibete	neg	pos	2	yes
MCAM/ET/2013/MU/11	Afar	Megalle	Adahara	pos	neg	3	yes
MCAM/ET/2013/MU/12	Afar	Yallo	Gubidera	pos	neg	3	no
MCAM/ET/2013/MU/13	Afar	Golina	Ullel-ella	pos	neg	3	yes
MCAM/ET/2013/MU/14	Afar	Awash Fentale	Alibete	neg	pos	3	yes
MCAM/ET/2013/MU/15	Afar	Awash Fentale	Dihoon	pos	neg	2	yes
MCAM/ET/2013/MU/16	Afar	Golina	Ullel-ella	pos	neg	2	no
MCAM/ET/2013/MU/17	Afar	Awash Fentale	Dihoon	pos	neg	2	yes
MCAM/ET/2013/MU/18	Afar	Megalle	Adahara	pos	neg	2	no
MCAM/ET/2013/MU/19	Afar	Megalle	Adahara	pos	neg	3	no
MCAM/ET/2013/MU/20	Afar	Golina	Ullel-ella	pos	neg	2	no
MCAM/ET/2013/MU/21	Afar	Megalle	Adahara	pos	neg	3	no
MCAM/ET/2013/MU/22	Afar	Megalle	Adahara	pos	neg	3	no

***In vitro* adaptation in HMI-9 medium with horse serum**

The highly concentrated trypanosome suspension in PSG was diluted to 2 x 10^5 cells ml^{-1} in Hirumi's modified Iscove's medium 9 (HMI-9), complemented with 15% (v/v) heat-inactivated foetal bovine serum (Gibco, Belgium) and 5% (v/v) heat-inactivated horse serum (Gibco, Belgium) (abbreviated as HMI-9 (HS)) (Van Reet *et al.* 2011; Hirumi & Hirumi 1989). Parasites were seeded at 2 x 10^4, 2 x 10^3 and 2 x 10^2 cells ml^{-1}, in a total volume of 500 µl in a 48-well plate (Nunc, Denmark) and incubated at 37 °C and 5% CO_2. After 72 hours, a well, where trypanosome density had increased above 2 x 10^5 cells ml^{-1}, was used for further subpassage in 500 µl of HMI-9 (HS). The well with the highest density of viable parasites was then further maintained in HMI-9 without horse serum (Van Reet *et al.* 2011). When possible, log phase growing *in vitro* cultures were scaled up in flasks (Nunc, Denmark) to obtain larger numbers of parasites for cryostabilisation, DNA extraction and *in vitro* drug sensitivity testing (Van Reet *et al.* 2014). The *in vitro* growth curves of the different stocks were generated by seeding cells at 1 x 10^4 cells ml^{-1} in 500 µl of HMI-9 in three replicate wells that were counted every 24 hours. The doubling times (T_d) were calculated from the exponential part of the curve using non-linear regression fitted with an exponential equation in GraphPad Prism 6 (GraphPad, version 6, USA).

Molecular characterisation of parasite populations

DNA extraction of trypanosome sediments prepared from the *in vivo* expanded and the *in vitro* adapted populations was performed with DNA Isolation Kit (Roche Diagnostics, Germany) following the protocol recommended for isolation of DNA from mammalian tissue. From *T.b. brucei* AnTat 1.1^E, *T.b. gambiense* LiTat 1.3, *T.b. gambiense* type II ABBA and *T. equiperdum* Dodola 940, DNA was extracted using the Maxwell 16 Tissue DNA Purification kit on a Maxwell 16 instrument according to the manufacturer's instructions (Promega, Belgium). DNA concentrations were measured using the Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, USA) and adjusted to 10 ng μl^{-1}. A set of PCRs targeting VSG genes (RoTat 1.2 and JN 2118Hu), maxicircle genes (ND4, ND5, ND7 and A6), class A minicircles (miniA PCR) and class B minicircles (EVAB PCR), minisatellites (MORF-2REP), P2 adenosine transporter (AT1) and the F1-ATP γ-subunit were adopted to characterise the studied parasite populations (Urakawa *et al.* 2001; Ngaira *et al.* 2005; Domingo *et al.* 2003; Dean *et al.* 2013; Njiru *et al.* 2006; Biteau *et al.* 2000; Graf *et al.* 2013). Where applicable, the published PCR protocols were adjusted to the requirements of the HotStarTaq Plus DNA polymerase (Qiagen, Germany). Primer sequences, reaction mixture contents, cycling conditions and expected amplicon size are described and referenced in Table 4.2. All PCR amplifications were carried out in 200 µl thin-wall PCR tubes (ABgene, UK) in a T3 thermocycler 48 (Biometra, Germany). Ten µl of amplified products were electrophoresed in 1 to 2% agarose gel at 135 V for 30 min and afterwards stained with ethidium bromide for visualization under UV light. For direct sequencing, PCR was performed in 50 – 100 µl volumes and amplicons were cleaned up and concentrated using a PCR cleanup kit (QIAquick PCR

Purification Kit, Qiagen, Germany) and sent out for bidirectional Sanger sequencing at the Genetic Sequencing Facility (VIB, Belgium) using the described PCR primers.

The full length sequence of the F1-ATP γ-subunit was cloned into a BamHI and HindIII double digested pHD309 vector using the In-Fusion Cloning kit (Clontech, Japan). Primers contained a F1-ATP γ-subunit specific sequence based on the *T. evansi* sequence of STIB 810 (EU185797) and a 5' extension of 15 bp specific to the place of integration in pHD309, containing the restriction sites and sequence overlap with the vector, as required for the In-Fusion Cloning reaction. Proofreading-PCR was performed using the Clone-Amp HiFi PCR premix (Clontech, Japan). Amplicons were cleaned up (QIAquick PCR Purification Kit, Qiagen, Germany) before use in the In-Fusion protocol. The reaction products were transformed in Stellar competent cells according to the manufacturer's recommendations (Clontech, Japan). Transformant clones were checked for the presence of insert using colony PCR, cultured in LB medium, plasmid purified (QIAprep Spin Miniprep Kit, Qiagen, Germany) and at least 7 to 12 clones per transformation were bidirectionally sequenced at the Genetic Sequencing Facility (VIB, Belgium) using primers binding to pHD309.

***In vitro* drug sensitivity testing**

Melarsomine dihydrochloride (Cymelarsan, Sanofi Aventis, France) and isometamidium hydrochloride (Veridium, Ceva Santé Animale, Belgium) were prepared as 10 mg ml^{-1} stock solutions in distilled water. Dophanil powder (Dophanil, Docpharma, Belgium), containing 445 mg diminazene diaceturate and 555 mg antipyrine per gram, was concentrated to a 10 mg ml^{-1} diminazene diaceturate solution in DMSO (Sigma, Belgium). Suramin (Germanin, Bayer, Germany) was prepared as a 100 mg ml^{-1} in DMSO. A method to measure the IC_{50} values of compounds in 96-well plates was performed as described elsewhere (Gillingwater *et al.* 2007). Briefly, 2×10^4 cells ml^{-1} from *in vitro* adapted stocks, each in four replicates, were exposed to seven threefold drug dilutions, ranging from 5000 to 7 ng ml^{-1} for suramin, 500 to 0.7 ng ml^{-1} for diminazene diaceturate and from 250 to 0.35 ng ml^{-1} for melarsomine dihydrochloride and isometamidium hydrochloride, in a total volume of 200 µl of HMI-9 medium. Next, the plate was incubated for 72 hours at 37°C with 5% CO_2 followed by addition of 20 µl of resazurin (Sigma, Belgium; 12.5 mg in 100 ml PBS) for measuring trypanosomes viability. After a further 24 h incubation at 37°C and 5% CO_2, fluorescence was measured (excitation λ = 560 nm; emission λ = 590 nm) with a VictorX3 multimodal plate reader using top reading (Perkin Elmer, Belgium) (Van Reet *et al.* 2014). The results were expressed as the percent reduction in parasite viability compared to the parasite viability in control wells without drugs. The 50% inhibitory concentration (IC_{50}) was calculated using non-linear regression fitted with a (log) inhibitor versus normalised response (variable slope) equation (GraphPad, version 6, USA). The IC_{50} values obtained from day 30 and day 60 *in vitro* cultures were compared using t-tests corrected for multiple testing according to the Holm-Sidak method ($\alpha = 0.05$) (GraphPad, version 6, USA).

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Table 4.2: PCRs used in the present study with target sequence, primer name and sequences, length of expected amplicon, reaction mixtures and cycling conditions. Reaction mixture 1: 25 µl containing 25 ng DNA, 1X CoralLoad buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 0.5 µM of each primer, 0.5 U of HotStar TaqPlus. Reaction mixture 2: 25 µl containing 25 ng DNA, 1X CoralLoad buffer, 1.5 mM of MgCl2, 200 µM of dNTPs, 1 µM of each primer, 0.5 U of HotStar TaqPlus. Reaction mixture 3: 25 µl containing 25 ng DNA, 1X CloneAmp HiFi PCR premix and 0.25 µM of each primer. bp: base pair, P: Plus DNA strand, M= Minus DNA strand.

Target sequence	Primers	Primer sequences	Amplicon length	Reaction mixture	Cycling conditions	Adapted from
VSG RoTat 1.2	ILO7957	5'-GCC ACC ACG GCG AAA GAC-3'	488 bp	1	95 °C for 5 min and 35 cycles of 30 sec at 94 °C, 30 sec at 58 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C	(Urakawa *et al.* 2001)
	ILO8091	5'-TAA TCA GTG TGG TGT GC-3'				
VSG JN 2118Hu	Forward	5'-TTCTACCAACTGACGGAGCG-3'	273 bp	1	95 °C for 5 min and 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C	(Ngaira *et al.* 2005)
	Reverse	5'-TAGCTCCGGATGCATCGGT-3'				
Maxicircle A6	Forward	5'-AAAAATAAGTATTTTGATATTATTAAAG-3'	381 bp	2	95 °C for 5 min and 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 30 s followed by a final elongation step at 72 °C for 8 min	(Domingo *et al.* 2003)
	Reverse	5'-TATTATTAACTTATTTGATC-3'				
Maxicircle ND4	Forward	5'-TGTGTGACTACCAGAGAT-3'	256 bp	2	Idem as above	(Domingo *et al.* 2003)
	Reverse	5'-ATCCTATACCCGTGTGTA-3'				
Maxicircle ND5	Forward	5'-TGGGTTTATATCAGGTTCATTTATG-3	400 bp	2	Idem as above	(Dean *et al.* 2013)
	Reverse	5'-CCCTAATAATCTCATCCGCAGTACG-3'				
Maxicircle ND7	Forward	5'-ATGACTACATGATAAGTA-3	167 bp	2	Idem as above	(Domingo *et al.* 2003)
	Reverse	5'-CGGAAGACATTGTTCTACAC-3'				

Minicircle class A	MiniA Reverse MiniB	5'-GGGTTTTTTAGGTCCGAG-3' 5'-CCGAAAATAGCACGTG-3'	1000 bp	1	95 °C for 5 min and 35 cycles of 30 sec at 94 °C, 30 sec at 58 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C	(Njiru *et al.* 2006)
Minicircle class B	EVAB1 EVAB2	5'-CACAGTCCGAGAGATAGAG-3' 5'-CTGTACTCTACATCTACCTC-3'	436 bp	1	95 °C for 5 min and 30 cycles of 30 sec at 94 °C, 30 sec at 60 °C, 60 sec at 72 °C and final extension for 10 min at 72 °C	(Njiru *et al.* 2006)
Minisatellite MORF2-REP	P M	5'TGCATGGCAATAGCGATGGGC-3' 5'ATCGTCACCTGGTGTACTTCTC-3'	repeated 102 bp sequence	1	95°C for 5 min fand 30 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 sec and extension at 72°C for 3 min. Elongation was continued for 72°C for 5 min	(Biteau *et al.* 2000)
TeAT1	TbAT1-F TbAT1-R-	5'-GAAATCCCCGTCTTTTCTCAC-3' 5'-ATGTGCTGAGCCTTTTTCCTT-3'	1600 bp	1	95 °C for 5 min, 24 cycles of 1 min at 95°C followed by 1 min at 56 °C and 2 min at 72 °C, and final extension at 72 °C for 5 min	(Graf *et al.* 2013)
F1-ATP synthase Y subunit	F1-ATP-F F1-ATP-R	5'-AACTGCAACGAAGCTTATGTCGGGCAAGCTTCGTC-3' 5'-TAAATGGGCAGGATCCCTACTTGGTTACTGCCCCTTC-3'	918 bp	3	98 °C for 30 sand 35 cycles of 98 °C for 15 s, 59.4 °C for 15 s , 72° C for 20 s and 72 °C for 5 min followed by cool down to 4 °C	

Microscopic examination for presence of a kinetoplast in trypanosomes

Trypanosome populations at different stages of *in vivo* and *in vitro* expansion were examined for the presence of the kinetoplast using 4',6-diamidino-2-phenylindole (DAPI) staining. Briefly, live trypanosomes in PSG or *in vitro* culture medium were washed in PBS by centrifugation, deposited onto microscope slides, air dried and fixed with methanol for 30 min. Subsequently, the slides were rehydrated in PBS and mounted in 87% glycerol containing 1 µg ml^{-1} DAPI (Sigma, Belgium) (Dean *et al.* 2013). Images were captured with an epifluorescence microscope (Olympus BX41, Olympus, Japan) equipped with a NU fluorescent cube (excitation: 360-370 nm and emission > 420 nm)) and Cell^D software (Olympus, Japan). DAPI stained trypanosomes were grouped according to the number of kinetoplasts (K) and nuclei (N) present within each cell. The percentage of kinetoplastic cells in a DAPI stained slide was calculated on the basis of on average 300 examined trypanosomes, by dividing the sum of 1K1N + 2K1N + 2K2N cells by the sum of 1K1N + 2K1N + 2K2N + 0K1N + 0K2N cells. A two-tailed Spearman correlation matrix (using a confidence interval of 95%) was used to find the correlation between the percentage of kinetoplastic cells at day 30 and day 60 of *in vitro* culture and the respective IC_{50} value for a particular drug (GraphPad, version 6, USA).

***In vivo* infectivity check**

To check the *in vivo* infectivity of trypanosome populations that were cryostabilised after continuous propagation *in vitro* for 60 days, 5 x 10^6 cells in 300 µl were inoculated in a single OF-1 mouse where after parasitaemia was checked as described above.

4. Results

Isolation of Ethiopian *T. evansi* stocks

Thirty cryopreserved buffy coat specimens from parasitologically positive dromedary camels were inoculated in immunosuppressed Swiss albino mice. In total, 22 parasite stocks originating from 22 different animals could be isolated and cryopreserved after 2 to 5 subpassages in mice. They were labelled as MCAM/ET/2013/MU/01 to MCAM/ET/2013/MU/22. Based on positivity in RoTat 1.2 PCR and EVAB PCR of the corresponding cryopreserved buffy coats, 20 of these stocks are *T. evansi* type A and 2 are *T. evansi* type B (Table 4.1) (Birhanu *et al.* 2015a). Copy cryovials of these primary isolates were brought to ITM, Antwerp and 14 were selected for further expansion in mice. The selection was based on their geographical origin and subtype: 12 type A stocks originated from different sampling stations in Afar and Tigray (MCAM/ET/2013/MU/01, 02, 04, 05, 06, 07, 08, 09, 11, 13, 15, 17) and two type B stocks (MCAM/ET/2013/MU/10 and 14) were from Awash Fentale in Afar. At peak parasitaemia, between 4 to 7 DPI, parasites were harvested, purified from blood using a mAECT column, washed with PSG and pelleted for DNA extraction and for *in vitro* culture adaptation.

Molecular typing based on specific VSG sequences of *in vivo* expanded stocks

DNA extracts of *in vivo* expanded stocks were subjected to RoTat 1.2 PCR and JN 2118Hu PCR to identify the *T. evansi* type based on type-specific VSG sequences. In addition, the specificity of these PCRs was tested on DNA of other *Trypanozoon* strains (*T.b. brucei* AnTat 1.1^{E}, *T.b. gambiense* LiTat 1.3, *T.b. gambiense* type II ABBA, *T. evansi* type A RoTat 1.2, *T. evansi* type B KETRI 2479 and *T. equiperdum* Dodola 940). Results are represented in Table 4.3. All the *in vivo* expanded stocks that originated from RoTat 1.2 PCR positive buffy coats, were also positive in RoTat 1.2 PCR (MCAM/ET/2013/MU/01, 02, 04, 05, 06, 07, 08, 09, 11, 13, 15 and 17). Direct sequencing of the 488 bp amplicons from these putative *T. evansi* type A stocks and the *T. evansi* RoTat 1.2 strain revealed 100% identity (in a 350 bp sequenced fragment) with the published RoTat 1.2 VSG sequence (AF317914), thus identifying them as *T. evansi* type A. Only one synonymous polymorphism (C699A) was found in MCAM/2013/ET/MU/04. The gel with the RoTat 1.2 PCR products from the purified trypanosomes showed a faint band of about 400 bp amplified in *T. evansi* KETRI 2479 and in MCAM/ET/2013/MU/10 and 14. Direct sequencing of these 400 bp amplicons failed, probably due to the low concentrations of the amplicons. The PCR targeting the *T. evansi* type B specific VSG JN 2118Hu generated the expected amplicon in *T. evansi* type B KETRI 2479 and in MCAM/ET/2013/MU/10 and 14. Additionally, an amplicon was generated from MCAM/ET/2013/MU/15. Also for *T.b. brucei* AnTat 1.1^{E} and *T.b. gambiense* type II ABBA, amplicons of 273 bp were produced in the JN 2118Hu PCR. Direct sequencing of these amplicons revealed that the Ethiopian *T. evansi* type B MCAM/ET/2013/MU/10 and 14, *T. evansi* type B KETRI 2479 and *T.b. brucei* AnTat 1.1^{E} were 100% identical (in a 190 bp sequenced fragment) to the corresponding sequence of JN 2118Hu VSG (AJ870486). In *T.b. gambiense* type II ABBA, one synonymous mutation (G300A) was found.

Table 4.3. Genetic characteristics of the trypanosome populations studied. pos = positive, neg = negative, seq = sequence identity, n.a. = not applicable, n.d. = not done, (f) = faint, * amplification failed may be due to restricted elongation time in PCR protocol or probably high number of repeats present.

	RoTat 1.2		JN 2118Hu		Maxicircle PCR				Minicircle class		Fraction of kinetoplastic cells *in vivo*	Minisatellite profile
Trypanosome stock or strain	**PCR**	**seq**	**PCR**	**seq**	**ND4**	**ND5**	**ND7**	**A6**	**A**	**B**	**%**	**MORF2-REP**
T.b. brucei AnTat 1.1[E]	neg	n.a.	pos	identical	pos	pos	pos	pos	pos (f)	neg	n.d.	neg*.
T.b. gambiense LiTat 1.3	neg	n.a.	neg	n.a.	pos	pos	pos	pos	neg	neg	n.d.	7,11 (f)
T. b. gambiense ABBA	neg	n.a.	pos	G300A	pos	pos	pos	pos	neg	neg	n.d.	3
T. evansi RoTat 1.2	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	97	4,6
T. evansi KETRI 2479	neg	n.a.	pos	identical	neg	neg	neg	neg	neg	pos	98	3,5
T. equiperdum Dodola 940	neg	n.a.	neg	n.a.	pos	pos	pos	pos	neg	neg	n.d.	11(f)
MCAM/ET/2013/MU/001	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	97	7
MCAM/ET/2013/MU/002	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	98	6,7
MCAM/ET/2013/MU/004	pos	C699A	neg	n.a.	neg	neg	neg	neg	pos	neg	99	6,7
MCAM/ET/2013/MU/005	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	98	6,7
MCAM/ET/2013/MU/006	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	99	6,7
MCAM/ET/2013/MU/007	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	100	6,7
MCAM/ET/2013/MU/008	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	98	6,7
MCAM/ET/2013/MU/009	pos	identical	neg	n.a.	neg	neg	neg	neg	neg	neg	0%	6,7
MCAM/ET/2013/MU/010	neg	n.a.	pos	identical	neg	neg	neg	neg	neg	pos	98	3,4
MCAM/ET/2013/MU/011	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	pos (f)	98	7
MCAM/ET/2013/MU/013	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	neg	99	6,7
MCAM/ET/2013/MU/014	neg	n.a.	pos	identical	neg	neg	neg	neg	neg	pos	99	3,4
MCAM/ET/2013/MU/015	pos	n.d.	pos	n.d.	neg	neg	neg	neg	pos	pos	98	3,4,6,7
MCAM/ET/2013/MU/017	pos	identical	neg	n.a.	neg	neg	neg	neg	pos	pos (f)	99	6,7

Morphological and genotypic kDNA status of the *in vivo* expanded stocks

Four PCRs that target maxicircle DNAs, of which three NADH-dehydrogenase subunits (ND4, ND5, ND7) and the ATPase subunit 6 (A6), and two PCRs that target class-specific minicircle sequences (miniA PCR and EVAB PCR) were run on DNA extracts of the purified trypanosomes (Table 4.3). All Ethiopian *T. evansi* stocks and *T. evansi* type A RoTat 1.2 and *T. evansi* type B KETRI 2479 were negative for all four maxicircle genes, while *T.b. brucei* AnTat 1.1^{E}, *T.b. gambiense* LiTat 1.3, *T.b. gambiense* type II ABBA and *T. equiperdum* Dodola 940 were positive for all four maxicircle genes.

All stocks that contain RoTat 1.2 VSG, except MCAM/ET/2013/MU/09, were positive in miniA PCR. Additionally, weak amplification was seen in *T.b. brucei* AnTat 1.1^{E}. MCAM/ET/2013/MU/10 and 14 were positive in EVAB PCR, confirming their identification as *T. evansi* type B as observed on their corresponding buffy coat specimens (Table 4.1). Additionally, EVAB PCR amplicons were detected in 3 stocks that were also positive for RoTat 1.2 VSG PCR suggesting a mixed infection with type A and B: a strong amplification was present in MCAM/ET/2013/MU/15, while a weak amplification was visible in MCAM/ET/2013/MU/11 and 17. The presence of kinetoplasts in the trypanosome cells was demonstrated using fluorescence microscopy with DAPI staining on *ex vivo* isolated trypanosomes (Table 4.3). *T. evansi* RoTat 1.2, *T. evansi* KETRI 2479 and all but one Ethiopian *T. evansi* stocks show a kinetoplast in > 96% of the cells. Stock MCAM/ET/2013/MU/09 was found to be akinetoplastic since only the nucleus of the trypanosomes was visible with DAPI.

MORF2-REP minisatellite profile of the *in vivo* expanded stocks

In *T. evansi* RoTat 1.2, the MORF2-REP locus consists of 4 and 6 repeats, while in *T. evansi* KETRI 2479, 3 and 5 repeats were found (Table 4.3). *In vivo* expanded Ethiopian stocks of type A had either 1 allele (7 repeats) or 2 alleles (6 and 7 repeats), thus displaying a different pattern than *T. evansi* type A RoTat 1.2. The Ethiopian type B stocks MCAM/ET/2013/MU/10 and 14 contain 3 and 4 repeats, and thus have a pattern different from *T. evansi* type B KETRI 2479. MCAM/ET/2013/MU/15 showed a clear pattern of the Ethiopian type B (3 and 4 repeats), and double allele pattern of the Ethiopian type A (6 and 7 repeats). The other presumed mixed type A and type B stocks MCAM/ET/2013/MU/11 and 17 showed only the Ethiopian type A *T. evansi* pattern (Fig 4.1). DNA extracted from the buffy coats revealed similar MORF2-REP patterns as the *in vivo* expanded trypanosomes except for the buffy coat of MCAM/ET/2013/MU/15 that revealed only the Ethiopian type A MORF2-REP pattern. The other *Trypanozoon* strains showed the following patterns: *T. b. gambiense* LiTat 1.3 had 7 and 11 repeats, *T.b. gambiense* type II ABBA had 3 repeats, *T. equiperdum* Dodola 940 had 11 repeats, while no amplicons were generated from *T.b. brucei* AnTat 1.1^{E} under the giving PCR conditions.

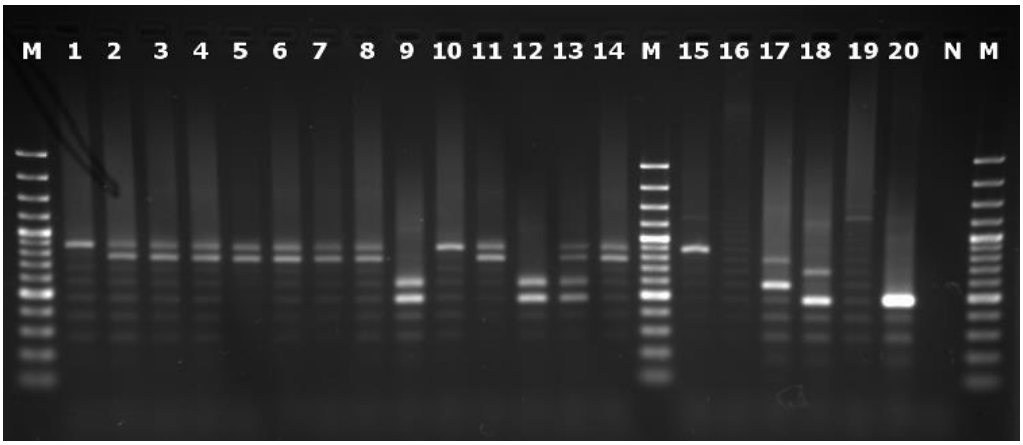

Figure 4.1. MORF2-REP profiles of Ethiopian *T. evansi* stocks and *T. evansi* and *T. brucei* reference strains. 1.5 % agarose gel showing MORF2-REP minisatellite PCR amplicons. Lane M: 100 bp plus marker, lanes 1 to 14: Ethiopian *T. evansi* stocks MCAM/ET/2013/MU/01-02-04-05-06-07-08-09-10-11-13-14-15-17, lane 15: *T.b. gambiense* LiTat 1.3, lane 16: *T.b. brucei* AnTat 1.1^{E} lane 17: *T. evansi* type A (RoTat 1.2), lane 18: *T. evansi* type B (KETRI 2479), lane 19: *T. equiperdum* Dodola 940, lane 20: *T. b. gambiense* ABBA, lane N: negative control.

F1-ATP synthase γ subunit genotyping

Sequence analysis of in total 136 clones of the full length F1-ATP synthase γ subunit, amplified from DNA of the *in vivo* expanded Ethiopian stocks MCAM/ET/2013/MU/04, 06, 09, 10, 11, 13, 14, 15 and of *T.b. brucei* AnTat 1.1^{E}, *T.b. gambiense* LiTat 1.3, *T. evansi* RoTat 1.2, *T. evansi* KETRI 2479, *T. b. gambiense* type II ABBA and *T. equiperdum* Dodola 940 revealed diverse homozygous and heterozygous nucleotide polymorphisms spread over the entire coding sequence (Table 4.4).

The F1-ATP synthase γ subunit of *T.b. gambiense* LiTat 1.3 (KT934830) appeared homozygous and identical to the *T.b. gambiense* DAL972 sequence (Tbg972.10.90). *T.b. gambiense* type II ABBA (KT934831) appeared homozygous and differed in only two SNPs (G801T and A882G) from the *T.b. gambiense* sequence. *T. evansi* RoTat 1.2 and the Ethiopian stocks MCAM/ET/2013/MU/04, 06, 09,11 and 13 were heterozygous and revealed in one allele (KT934833), identical to the published full length *T. evansi* STIB 810 (EU185798) sequence, the deletion of nucleotides A841-843del. The second allele contained a C142T polymorphism (KT934832), that is not present in the wild-type *T. evansi* STIB 810 sequence (EU185797), but that could be identified in the genome sequence of the Chinese akinetoplastic *T. evansi* STIB 805 strain (Carnes *et al.* 2015). For *T. evansi* KETRI 2479 and the Ethiopian stocks MCAM/ET/2013/MU/10 and 14 we obtained heterozygous alleles, different from the partial sequence of *T. evansi* KETRI 2479 (EU185794). The first allele had the unique A844T polymorphism (KT934835), and differed from the second allele in 3 additional SNPs (T321C,

T807C, T867G) that were also found in some *T.b. brucei* and *T. equiperdum*. Interestingly, the *in vivo* expanded stock of MCAM/ET/2013/MU/15 revealed alleles that belonged to *T. evansi* type A and type B. In contrast, when the original buffy coat of this stock was tested, only alleles of *T. evansi* type A were found. Finally, *T. equiperdum* Dodola 940 (KT934836) appeared homozygous and its single allele was identical to one of the two alleles found in *T.b. brucei* AnTat 1.1^{E} (KT934837), but differed in 5 SNPs with the sequence from *T. equiperdum* BoTat 1.1 (EU185793) and in 6 SNPs with *T. equiperdum* STIB 841 (EU185792). However, for the *T. equiperdum* STIB 841 strain, 5 of the 6 SNPs were ambiguous polymorphisms that do not rule out similarity to *T. equiperdum* Dodola 940.

***In vitro* adaptation of Ethiopian *T. evansi* stocks**

Fourteen Ethiopian *T. evansi* stocks, *T. evansi* RoTat 1.2 and *T. evansi* KETRI 2479 were expanded in mice and purified from blood at peak parasitaemia to initiate primary *in vitro* cultures in HMI-9 (HS) medium. After 96 hours, the initial $2x10^{4}$ cells ml^{-1} inoculum reached concentrations above $2x10^{5}$ cells ml^{-1} for all the different stocks. These cells were used for further *in vitro* propagation by subpassage in fresh medium. Over the next 72 hours, only MCAM/ET/2013/MU/09, 14 and 15, and *T. evansi* RoTat 1.2 and *T. evansi* KETRI 2479 showed proliferation. In contrast, slightly increased cell densities were observed for MCAM/ET/2013/MU/01, 04, 06 and 10. For all other strains not a single inoculum proliferated and longer incubation led to growth cessation.

Because the HMI-9 (HS) medium did not support sufficient *in vitro* culture growth for most of the Ethiopian *T. evansi* stocks, it was abandoned and replaced with HMI-9 without horse serum. *In vitro* adapted strains of *T.b. brucei* AnTat 1.1^{E} and *T.b. gambiense* LiTat 1.3 were cultured in HMI-9 in parallel. *In vitro* cultures were only considered adapted to HMI-9 medium when it was possible to maintain the parasites in continuous proliferation. To this extent, dense parasite cultures, containing $2 – 5 \times 10^{5}$ cells ml^{-1}, were subpassaged into new wells using serial fivefold dilutions in fresh medium. When these subpassages reached densities above 2×10^{5} cells ml^{-1} within a 48 - 96 hours period, the stock was considered adapted. The five stocks that already grew well in the HMI-9 (HS) medium continued proliferating when inoculated from the dense cultures at serial fivefold dilutions in HMI-9. These five stocks were considered to be *in vitro* adapted after 15 days of *in vitro* culture. Out of the four remaining stocks, only MCAM/ET/2013/MU/04 and 10 slowly regained the ability to proliferate in HMI-9 at a reduced subpassaging scheme using serial twofold dilutions. MCAM/ET/2013/MU/04 required 25 days to adapt, while MCAM/ET/2013/MU/10 was only fully adapted after day 35 of *in vitro* culture. Gradually increasing the culture volume allowed to obtain sufficient parasites from the adapted cultures for *in vitro* drug testing, DNA extraction, and cryostabilisation at day 30 (all, except MCAM/ET/2013/MU/10) and at day 60 of *in vitro* culture (all stocks).

Table 4.4: F1-ATP synthase γ subunit single nucleotide polymorphism (SNP) observed within the studied trypanosome stocks and strains or retrieved from GenBank. Some SNPs were only present in *T.b. b.* TREU927 (G6A, C9T,C572G), T.b.b 29-13 (C149G, A168C, C866T) and *T.b.b*. STIB 920 (G738C) and are not represented in the table. del=deletion, GAN: GenBank accession number, * identical to all Ethiopian *T. evansi* type A stocks, ** identical to all Ethiopian *T. evansi* type B stocks. Blank spaces indicate no change and – indicates missing sequence information.

Stock/strain	A93G	C142T	C194T	A198G	G294A	T321C	A356T	T654C	T663C	G801T	T807C	G817C	841-843 GCT	A844T	T867G	A882G	T892C	GAN
T.b.g. DAL972	G			G						T						G		Tbg972.10.90
T.b.g. LiTat 1.3	G			G						T						G		KT934830
T.b.g. ABBA	G			G														KT934831
T. ev. RoTat 1.2*		T	T															KT934832
T. ev. RoTat 1.2*			T										del					KT934833
T. ev. STIB 810			T															EU185797
T. ev. STIB 810			T										del					EU185798
T. ev. KETRI 2479**						C					C				G			KT934834
T. ev. KETRI 2479**														T				KT934835
T. ev. KETRI 2479	-	-	-	-	-	-	-	-	-		C			T	G			EU185794
T. eq BoTat 1.1						C					C	C						EU185793
T. eq. STIB 841			Y		R	C	W	Y	Y						G		C	EU185792
T. eq. Dodola 940			T			C			C						G			KT934836
T.b.b. AnTat 1.1^{E}			T			C			C						G			KT934837
T.b.b. AnTat 1.1^{E}					A				C									KT934838
T.b.b. STIB 920						C			C		C				G			EU185791
T.b.b. 29-13	-		T			C			C						G		C	EU185790
T.b.b. TREU927	G				A	C	T	C										Tb927.10.180

DNA of the *in vitro* adapted stocks was subjected to RoTat 1.2 PCR, EVAB PCR and MORF2-REP PCR. All *in vitro* stocks had similar molecular profiles as their corresponding *in vivo* expanded parental stocks, except MCAM/ET/2013/MU/15. While the *in vivo* expanded stock of the latter was identified as a mixed infection of *T. evansi* type A and type B, the *in vitro* adapted stock (at day 30 and day 60 *in vitro* culture) was identified as pure *T. evansi* type B with the above mentioned PCRs and confirmed by cloning and sequencing of the F1-ATP synthase γ subunit. Thus, beside *T. evansi* RoTat 1.2 and *T. evansi* KETRI 2479, we achieved the *in vitro* adaptation of 2 Ethiopian type A stocks, 2 Ethiopian type B stocks and additionally ended up with a pure *T. evansi* type B *in vitro* adapted stock originating from a mixed type A and type B *in vivo* adapted stock. Growth curves were generated for *T.b. brucei* AnTat 1.1^{E} and all seven *in vitro* adapted stocks (Fig.4.2). *T.b. brucei* AnTat 1.1^{E} and *T. evansi* RoTat 1.2 had the shortest T_d, 7.5 ± 0.3 h^{-1} and 7.7 ± 0.2 h^{-1} respectively, and reached the highest maximum population density (MPD) of ± 3 – 4 x 10^6 cells ml^{-1}, while *T. evansi* KETRI 2479 had a longer T_d, 10.8 ± 0.2 h^{-1}, and a lower MPD of ± 1 x 10^6 cells ml^{-1}. The Ethiopian type A stocks MCAM/ET/2013/MU04 and MU09 had a T_d of 11.2 ± 0.4 and 11.3 ± 0.4 respectively, and a MPD of ± 1 x 10^6 cells ml^{-1}. Similarly, the Ethiopian type B stocks MCAM/ET/2013/MU10, 14 and 15 had a T_d of 12.9 ± 0.5, 11.3 0.5 and 12.1 ± 0.6 respectively, and a MPD of ± 0.7 – 1 x 10^6 cells ml^{-1} (Fig 4.2).

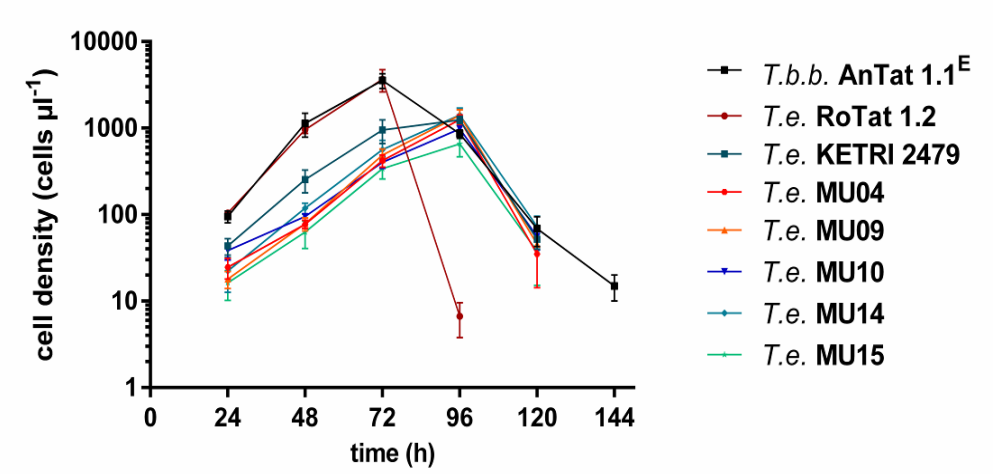

Figure 4.2: *In vitro* growth curve of trypanosome stocks and strains. *T.b.b.* = *T.b. brucei*, *T.e.* = *T. evansi*, MU = MCAM/ET/2013/MU.

***In vitro* drug sensitivity and relation to kDNA**

After day 30 and 60 of *in vitro* culture, IC_{50} values were determined for melarsomine dihydrochloride (Cymelarsan) (Figure 4.3A), isometamidium hydrochloride (Veridium) (Figure 4.3B), diminazene diaceturate (Dophanil) (Figure 4.3C) and suramin (Germanin) (Figure 4.3D). In general, non-significant differences ($p > 0.05$) were found between IC_{50} values recorded at day 30 and day 60 of *in vitro* culture, except for the melarsomine dihydrochloride IC_{50} values of *T. evansi* RoTat 1.2 and *T. evansi* MCAM/ET/2013/MU/14 and for the isometamidium hydrochloride IC_{50} values of *T. evansi* KETRI 2479 and *T. evansi* MCAM/ET/2013/MU/09 ($p < 0.05$). For comparison between the different stocks, the IC_{50} values of day 30 and day 60 of *in vitro* cultures were averaged. All Ethiopian *T. evansi* stocks had IC_{50} values for melarsomine dihydrochloride (IC_{50} 1.9 – 3.3 ng ml^{-1}) that were similar to those of *T.b. gambiense* LiTat 1.3 (IC_{50} 4.3 ng ml^{-1}), *T.b. brucei* AnTat 1.1^{E} (IC_{50} 6.8 ng ml^{-1}), *T. evansi* RoTat 1.2 (IC_{50} 3.0 ng ml^{-1}) and *T. evansi* KETRI 2479 (IC_{50} 4.1 ng ml^{-1}). For isometamidium hydrochloride, the IC_{50} values of the Ethiopian *T. evansi* (IC_{50} 0.6 – 6.2 ng ml^{-1}) fall within the range of *T.b. gambiense* LiTat 1.3 (IC_{50} 0.1 ng ml^{-1}), *T.b. brucei* AnTat 1.1^{E} (IC_{50} 7.3 ng ml^{-1}), *T. evansi* RoTat 1.2 (IC_{50} 7.1 ng ml^{-1}) and *T. evansi* KETRI 2479 (IC_{50} 5.5 ng ml^{-1}). However, the two Ethiopian *T. evansi* type A stocks (IC_{50} 4.3 – 6.2 ng ml^{-1}) appear to be threefold less sensitive that the three type B stocks (IC_{50} 0.6 – 1.9 ng ml^{-1}). For suramin, large differences in IC_{50} values were found among the Ethiopian *T. evansi* (IC_{50} 15.9 – 261.5 ng ml^{-1}) stocks and among the other strains: *T.b. brucei* AnTat 1.1^{E} (IC_{50} 39.5 ng ml^{-1}) and *T. evansi* RoTat 1.2 (IC_{50} 35.8 ng ml^{-1}) appear highly susceptible, while *T.b. gambiense* LiTat 1.3 (IC_{50} 134.0 ng ml^{-1}) and *T. evansi* KETRI 2479 (IC_{50} 222.4 ng ml^{-1}) are less susceptible.

The two Ethiopian *T. evansi* type A (IC_{50} 153.5 – 261.5 ng ml^{-1}) appear to be tenfold less sensitive than the three type B (IC_{50} 15.9 – 27.6 ng ml^{-1}). For diminazene diaceturate, the IC_{50} values of all Ethiopian *T. evansi* (IC_{50} 17.5 – 48.5 ng ml^{-1}) are higher than those of *T.b. gambiense* LiTat 1.3 (IC_{50} 5.2 ng ml^{-1}) and *T. evansi* RoTat 1.2 (IC_{50} 13.8 ng ml^{-1}), but similar to *T.b. brucei* AnTat 1.1^{E} (IC_{50} 39.6 ng ml^{-1}) and *T. evansi* KETRI 2479 (IC_{50} 24.0 ng ml^{-1}). The two Ethiopian *T. evansi* type A (IC_{50} 37.4 – 48.5 ng ml^{-1}) appear to be twofold less sensitive than the three type B (IC_{50} 17.5 – 25.9 ng ml^{-1}). Direct sequencing of the full length TeAT1 PCR amplicons of MCAM/ET/2013/MU/04, 09, 10, 14, and 15, *T. evansi* type A RoTat 1.2 and *T. evansi* Type B KETRI 2479 revealed no polmorphisms to the wild-type *TeAT1* sequence (AB124588).

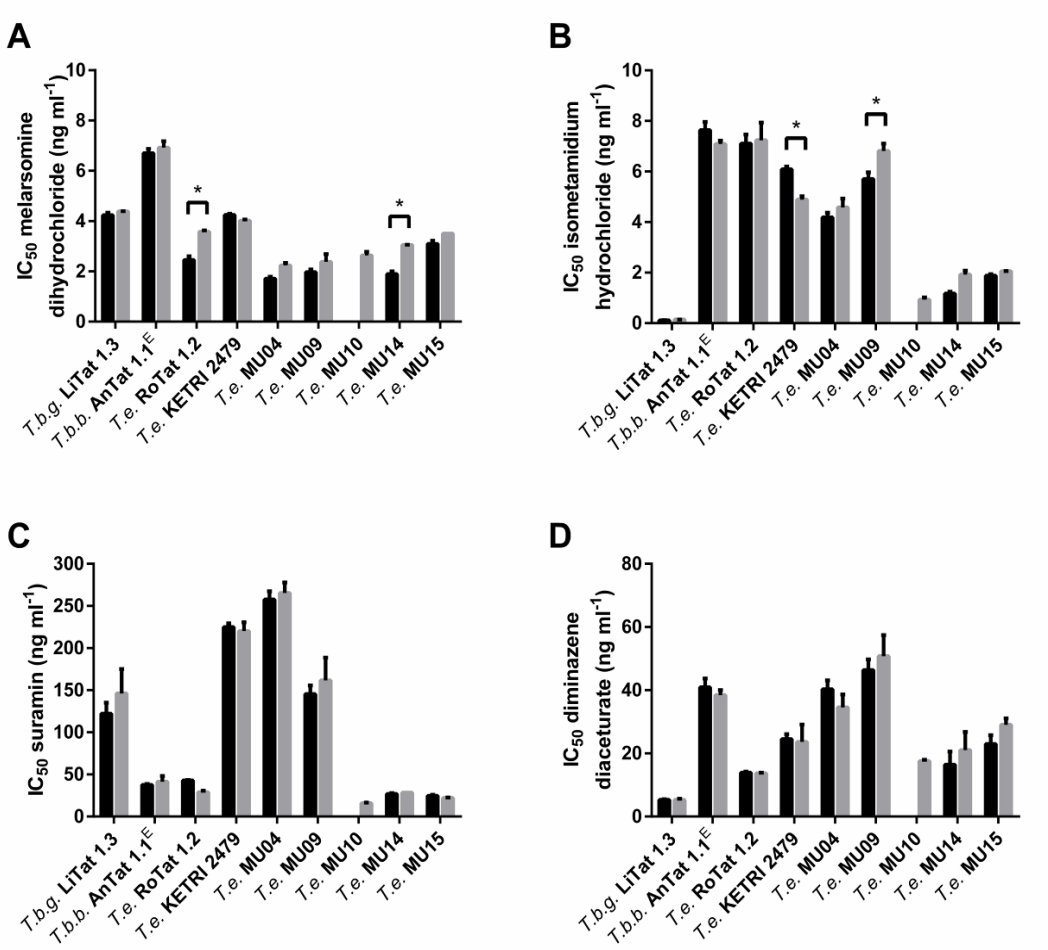

Figure 4.3: *In vitro* drug sensitivity of *T. evansi.* IC_{50} values for different drugs, with standard error bars, obtained with different *Trypanosoma sp*. stocks and strains after 30 (black bars) and 60 days (grey bars) *in vitro* culture. Significant differences between IC_{50} values of 30 and 60 days *in vitro* cultures are indicated by an asterisk. *T.b.g. = T.b. gambiense*, *T.b.b. = T.b. brucei*, *T.e. = T. evansi*, MU = MCAM/ET/2013/MU. Panel A: IC_{50} values for melarsomine dihydrochloride. Panel B: IC_{50} values for isometamidium hydrochloride. Panel C. IC_{50} values for suramin and Panel D: IC_{50} values for diminazene diaceturate.

DAPI staining was performed on *in vivo* and *in vitro* propagated stocks (Figure 4.4). *In vitro* culture did not change the percentage of kinetoplastic cells in *T.b. gambiense* LiTat 1.3 (99%), *T.b. brucei* AnTat 1.1^{E} (99%) and MCAM/ET/2013/MU/09 (0%) (Figure 4.5). On the other hand, already after 30 days *in vitro* culture a decrease in the percentage of kinetoplastic cells was

observed in *T. evansi* RoTat 1.2 (89%), *T. evansi* KETRI 2479 (81%), MCAM/ET/2013/MU/04 (97%), 14 (93%) and 15 (94%) compared to non-*in vitro* adapted trypanosomes. After 60 days of *in vitro* culture, the percentage of kinetoplastic cells dropped even further for *T. evansi* KETRI 2479 (64%), MCAM/ET/2013/MU/04 (89%) and 10 (35%). No significant correlation was observed between the percentage of kinetoplastid cells of all *in vitro* adapted *T. evansi* stocks (including day 30 and day 60) and their IC_{50} values for melarsomine dihydrochloride (ρ = -0.13, p = 0. 67), isometamidium hydrochloride (ρ = -0.324, p = 0.278), suramin (ρ = -0.097, p = 0.752) and diminazene diacetureate (ρ = -0.355, p = 0.233). These data suggest that among the *in vitro* adapted Ethiopian *T. evansi* stocks there is no relation between the drug sensitivity and the presence of kinetoplast DNA. Furthermore, their loss of kDNA does not seem to influence rodent infectivity since all cryostabilates made from day 60 *in vitro* cultures remained infective for mice with detectable parasitaemia at 4-5 DPI.

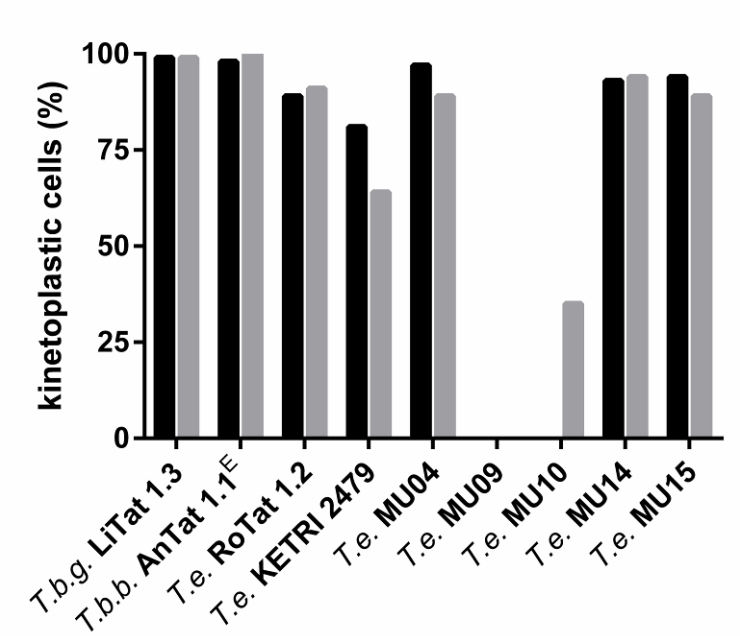

Figure 4.4. Percentage of kinetoplastic cells within *T. evansi* populations. Percentage of kinetoplastic cells visualised after DAPI staining and fluorescence microscopy within populations after 30 (black bars) and 60 days (grey bars) *in vitro* propagation. *T.b.g*. = *T.b. gambiense*, *T.b.b.* = *T.b. brucei*, *T.e*= *T. evansi*, MU = MCAM/ET/2013/MU.

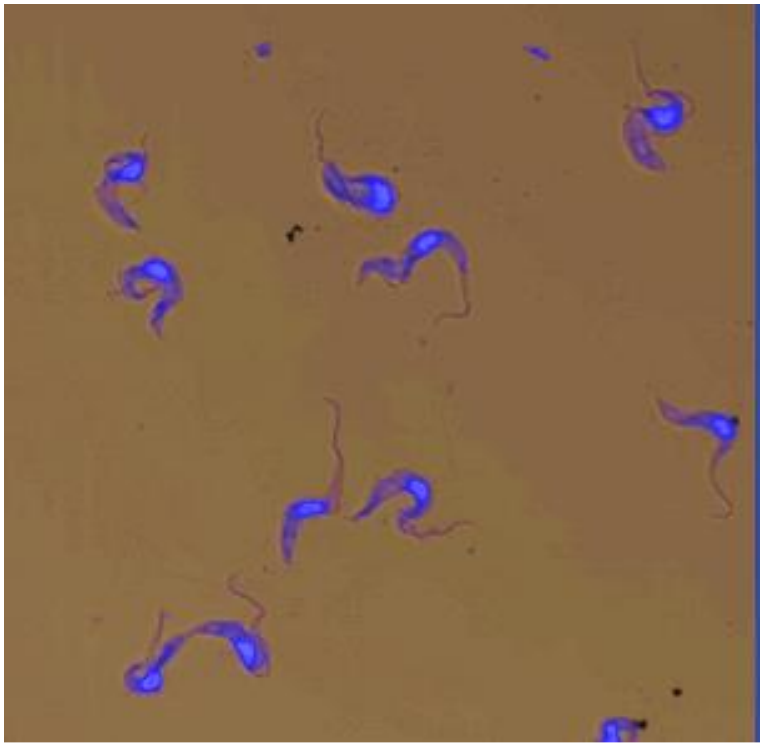

Figure 4.5: DAPI staining and confocal microscopy on *T. evansi* MCAM/ET/2013/MU/09 showing only the nucleus but no kinetoplast.

5. Discussion

Previous molecular and serological studies revealed that trypanosome infections in camels from Northern Ethiopia are caused by either RoTat 1.2 PCR or EVAB PCR positive parasites. In some instances amplicons of both PCRs were detected within the same buffy coat extract, suggesting the occurrence of mixed infections (Chapter 3, (Birhanu *et al.* 2015a)). The present study was undertaken to isolate the trypanosomes from camels carrying apparent single infections through inoculation of their buffy coats in immunosuppressed mice. The *in vivo* inoculation led to the successful isolation of 22 stocks, out of which 14 were selected on the basis of their geographical origins for further investigations (5 stocks from Tigray and 9 stocks from Afar). Next, we performed an in-depth comparative molecular analysis on DNA extracts from the isolated parasite stocks using diverse PCRs. Furthermore, we analysed the specificity of each of these PCRs on a collection of *Trypanozoon* strains.

The RoTat 1.2 VSG sequence can be used to characterise *T. evansi* type A (Urakawa *et al.* 2001; Claes *et al.* 2004). In our collection, all buffy coats positive in RoTat 1.2 PCR yielded *in vivo* isolated stocks that were RoTat 1.2 PCR positive but that were negative in the maxicircle gene

targeting PCRs. Furthermore, with the exception of the akinetoplastic stock MCAM/ET/2013/MU/09, all these strains had type A minicircles. MCAM/ET/2013/MU/09 may be naturally akinetoplastic since the DNA extracted from the original buffy coat was negative in all PCRs targeting kinetoplast DNA. The occurrence of naturally akinetoplastic strains was previously documented in Latin America and China (Stevens *et al.* 1989; Ventura *et al.* 2000; Ou *et al.* 1991; Lun & Vickerman 1991). One stock (MCAM/ET/2013/MU/04) contained a SNP in its RoTat 1.2 VSG PCR amplicon. SNPs in RoTat 1.2 amplicons were previously reported in Egypt but do not necessarily lead to a negative result in RoTat 1.2 based antibody detection tests. This was also the case for the camel from which MCAM/ET/2013/MU/04 was isolated (Elhaig *et al.* 2013; Amer *et al.* 2011).

Initially defined by minicircle class B, identification of *T. evansi* type B is possible with EVAB PCR that amplifies a fragment of this minicircle (Njiru *et al.* 2006). Additionally, it was proposed that the VSG JN 2118Hu, first described in a Kenyan *T. evansi* strain, is a specific marker for *T. evansi* type B (Ngaira *et al.* 2005).

In our collection, two buffy coat extracts that were positive in EVAB PCR yielded *in vivo* isolated stocks that were EVAB PCR positive as well. Interestingly, an EVAB PCR amplicon was also detected in three additional *in vivo* expanded stocks that were RoTat 1.2 PCR positive but for which the corresponding buffy coats were EVAB PCR negative. These three stocks might be mixed infections. JN 2118Hu VSG PCR appeared to be less sensitive because it detected only 3 out of 5 EVAB PCR positive isolated stocks. Furthermore, the JN 2118Hu VSG PCR appeared to be less specific since *T.b. brucei* AnTat 1.1^{E} and *T.b. gambiense* type II ABBA were also positive in this PCR. None of the EVAB PCR positive isolated stocks contained maxicircle DNA and they were all negative in miniA PCR, except for the three mixed infections. Therefore, we conclude that we isolated at least two “pure” *T. evansi* type B stocks from Ethiopian camels, decades after the initial isolation of *T. evansi* type B from camels in Kenya (Njiru *et al.* 2006).

We used the minisatellite locus MORF2-REP to verify whether both putative mixed stocks, that were positive in RoTat 1.2 PCR and EVAB PCR, were real mixed infections or hybrids between *T. evansi* type A and B. The Ethiopian isolates clustered in two classes of *T. evansi* type A, of which one with a previously described heterozygous profile (6 and 7 repeats) and one with a homozygous profile (7 repeats). The Ethiopian *T. evansi* type B stocks had a heterozygous profile (3 and 4 repeats) differing from the only known profile described for Kenyan type B isolates (3 and 5 repeats) (Masiga *et al.* 2006). In one of the mixed infections we observed a profile that can be interpreted as a mixture of Ethiopian type A and type B, while the others only revealed the Ethiopian type A pattern. These results prove that we are dealing with mixed infections and not with hybrids between *T. evansi* type A and type B. To exclude that these apparent mixed infections represent cross-contamination with genetic material, we attempted *in vitro* cultivation of the *in vivo* expanded stocks.

Previously we have shown that addition of 1,1% methylcellulose to HMI-9 greatly helps the *in vitro* adaptation of *Trypanozoon* strains, including *T.b. gambiense* and *T. evansi* RoTat 1.2 (Van Reet *et al.* 2011). However, to avoid the use of this highly viscous medium we preferred the use of horse serum to adapt *T. evansi* stocks, as suggested in previous reports (Hirumi *et al.* 1997; Kaminsky & Brun 1998; Zweygarth & Röttcher 1986). While this approach proved to be successful for all type B stocks, only two out of nine Ethiopian *T. evansi* type A could be adapted. Interestingly, in the case of mixed stock MCAM/ET/2013/MU/15, this medium selected *T. evansi* type B out of the mixed population. While only the type A infection was detected in the buffy coat DNA extract, both types could be detected in the *in vivo* expanded stock DNA, but eventually only type B was detected in the *in vitro* adapted stock.

Gillingwater and colleagues reported on the drug sensitivity profiles of a panel of *T. evansi* and *T. equiperdum* strains where they considered *T. evansi* STIB 806K to be a reference sensitive strain for suramin (IC_{50} 70.4 ng ml^{-1}), diminazene diaceturate (IC_{50} 4.5 ng ml^{-1}) and melarsomine dihydrochloride (IC_{50} 1.4 ng ml^{-1}). They reported drug resistance in two *T. evansi* stocks with an IC_{50} for suramin > 10000 ng ml^{-1} (STIB 780 and STIB 781), and in the *T. equiperdum* OVI strain, with an IC_{50} for diminazene diaceturate of 302 ng ml^{-1} and an IC_{50} for melarsomine dihydrochloride of 17.6 ng ml^{-1} (Gillingwater *et al.* 2007). The only strain that is shared between their panel and our collection is *T. evansi* RoTat 1.2, which despite different approaches in the experimental testing, yielded corresponding IC_{50} values, especially for diminazene diaceturate and melarsomine dihydrochloride, thus facilitating comparison between both studies. In our Ethiopian *T. evansi* collection, no resistance against melarsomine dihydrochloride was found. However, some stocks appeared to have raised IC_{50} values for suramin (> 200 ng ml^{-1}) and diminazene diaceturate (> 50 ng ml^{-1}). The IC_{50} values that we observe for *T.b. gambiense* LiTat 1.3 and the Ethiopian *T. evansi* type B are similar to the *in vitro* IC_{50} value of 0.82 ng ml^{-1} found by Sahin and coworkers for *T. congolense* IL3000 which is sensitive to isometamidium (Veridium) *in vivo* (Sahin *et al.* 2014). In the same study, an *in vitro* IC_{50} of 11.06 ng ml^{-1} is reported for *T.b. brucei* AnTat 1.1 strain, which is slightly higher than the value that we obtained in experiments with our *T.b. brucei* AnTat 1.1 strain and the other *T. evansi* stocks (Sahin *et al.* 2014). Nevertheless, defining our *T. evansi* stocks as either sensitive or resistant based solely on the *in vitro* drug sensitivity results may be too audacious, given the fact that IC_{50} values were determined in only one assay, the resazurin viability assay (Kaminsky *et al.* 1997; Räz *et al.* 1997; Van Reet *et al.* 2013). Therefore, an *in vivo* drug sensitivity profile of all our *Trypanozoon* strains against the commonly used trypanocides remains to be elucidated. Interestingly, both Ethiopian *T. evansi* type A stocks appear to be less susceptible to suramin, diminazene diaceturate and isometamidium hydrochloride than the three type B stocks. In *T.b. brucei*, resistance against suramin and isometamidum hydrochloride has been linked to several proteins (Alsford *et al.* 2012; Baker *et al.* 2015), while resistance to diamidine and melaminophenyl classes of drugs is attributed to the transporter protein TbAT1 and the aquaporin AQP2 (Munday *et al.* 2015a;

Munday *et al.* 2015b; Munday *et al.* 2014). The lower sensitivity to diminazene diaceturate was not caused by mutations in the *T. evansi TeAT1* (Witola *et al.* 2004).

Interestingly, DAPI staining of the trypanosomes indicated slight to severe loss of the kDNA in all *in vitro* adapted *T. evansi* stocks, when compared to *in vivo* adapted stocks. The loss of kDNA in *in vitro* cultured *T. evansi* is a phenomenon that has been known for a long time (Schnaufer *et al.* 2002; Njiru *et al.* 2006; Zweygarth *et al.* 1990; Kaminsky *et al.* 1997). Non-vital loss of the kinetoplast is made possible by mutations in the F1-ATP γ subunit of *T. evansi* allowing to uncouple from the Fo subunit and effectively circumventing the requirement for mitochondrial gene expression (Schnaufer *et al.* 2005). Furthermore, it has been shown that the expression of certain *T. evansi* F1-ATP γ subunit coding sequences in *T. brucei* allows this species to survive loss of its kDNA after chemical treatment (Dean *et al.* 2013). Moreover, in such genetically modified *T. brucei,* independence of kDNA maintenance and expression is associated with multidrug resistance (Gould & Schnaufer 2014). In our collection of *T. evansi* stocks we did not observe differences in drug sensitivity between populations that were partially or completely akinetoplast confirming earlier evidence that the presence or absence of kDNA is irrelevant within this context (Gould & Schnaufer 2014; Kaminsky *et al.* 1997).

Recently, Carnes *et al.* showed that SNPs in the F1- ATP γ subunit could be used to genotypically support the multiple origins of at least four dyskinetoplastic *T. evansi/T. equiperdum* lineages: one major group of RoTat 1.2 VSG positive *T. evansi/T. equiperdum* type A, and three very small groups each represented by only a single strain: *T. evansi* type B KETRI 2479, *T. equiperdum* BoTat and *T. equiperdum* OVI (Carnes *et al.* 2015). All Ethiopian *T. evansi* type A had the corresponding mutation of the type A group. The Ethiopian type B *T. evansi* shared a similar profile as KETRI 2479. Finally, the Ethiopian *T. equiperdum* strain Dodola, which had some maxicircle genes but was negative for both type A and type B markers revealed an F1-ATP synthase sequence similar to *T.b. brucei* AnTat 1.1^{E} strain, thus likely belongs to the same dyskinetoplastic group as *T. equiperdum* OVI (Dean *et al.* 2013; Carnes *et al.* 2015).

Summarizing, our study shows that the apparent *T. evansi* type that is detected in a buffy coat of an infected camel does not necessarily represent the full diversity that is present in the infected animal. Moreover, the fact that 5 out of 22 new *T. evansi* isolates from camel in Ethiopia contain *T. evansi* type B may be an indication that is more widespread than currently known. The inoculation of the trypanosomes in immunosuppressed mice may allow the propagation of mixed populations. In contrast, *in vitro* cultivation seems to reduce the diversity by selecting for only one particular type, in our study *T. evansi* type B. Secondly, our study addresses some drawbacks of current molecular markers for *T. evansi* genotyping. To rely solely on VSG markers or kDNA markers for the molecular identification of *T. evansi* may be misleading due to possible recombinations occurring in VSG genes and to the presence of akinetoplastic *T. evansi* stocks. In this regard, we confirm that the F1-ATP γ-subunit gene, that is not related to the VSG repertoire nor to the presence of kDNA, may become an interesting target for genotyping *T. evansi* stocks in

areas where both types overlap and where mixed infections can occur. Nevertheless, it is not possible to separate the Ethiopian *T. equiperdum* from *T. brucei* on the basis of this target gene. Thirdly, no evidence of *in vitro* drug resistance was found in our collection of *T. evansi* type A and type B stocks. The presence or partial absence of kDNA in the *in vitro* adapted *T. evansi* stocks did not correspond with the drug sensitivity phenotype.

From the above, we conclude that the presence of *T. evansi* type B in Ethiopian camels must be taken into account when proposing control measures against surra. For serological as well as for molecular screening, tests or test combinations that are able to detect both *T. evansi* type A and B should be used. The present data on *in vitro* drug sensitivity of both types do not suggest that it is necessary to differentiate type A from type B in order to choose the drug for treating infected animals.

In the epidemiological survey described in Chapter 3, we used the CATT/*T. evansi* for serological screening. In the meantime, the first immunochromatography test for *T. evansi* had been developed, the Surra Sero *K*-SeT. The major advantages of this test are its long term thermostability and its individual test format. In Chapter 5, we describe the diagnostic evaluation of the Surra Sero *K*-SeT on archived sera as a first step in the process of its further development and eventual implementation.

Surra Sero *K*-SeT, a new immunochromatographic test for serodiagnosis of *Trypanosoma evansi* infection in domestic animals

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Author contributions: HB and PB conceived and designed the experiments. HB and RB performed the experiments. HB, RB, PB, BMG and TG contributed to reagents, materials and analysis tools: SR, TS and PB developed the test. HB and PB analysed the data and wrote the manuscript. All authors revised and approved the final manuscript.

1. Abstract

Trypanosoma evansi, the causative agent of surra, infects different domestic and wild animals and has a wide geographical distribution. It is mechanically transmitted mainly by haematophagous flies. Parasitological techniques are commonly used for the diagnosis of surra but have limited sensitivity. Therefore, serodiagnosis based on the detection of *T. evansi* specific antibodies is recommended by the World Organisation for Animal Health (OIE). Recently, we developed a new antibody detection test for the serodiagnosis of *T. evansi* infection, the Surra Sero *K*-SeT. Surra Sero *K*-SeT is an immunochromatographic test (ICT) that makes use of recombinant variant surface glycoprotein rVSG RoTat 1.2, produced in the yeast *Pichia pastoris*.

In this study, we compared the diagnostic accuracy of the Surra Sero *K*-SeT and the Card Agglutination Test for *T. evansi* Trypanosomososis (CATT/*T. evansi*) with immune trypanolysis (TL) as reference test on a total of 806 sera from camels, water buffaloes, horses, bovines, sheep, dogs and alpacas. Test agreement was highest between Surra Sero *K*-SeT and TL (κ=0.91, 95% CI 0.841-0.979) and somewhat lower between CATT/*T. evansi* and TL (κ=0.85, 95% CI 0.785-0.922) and Surra Sero *K*-SeT and CATT/*T. evansi* (κ=0.81, 95% CI 0.742-0.878). The Surra Sero *K*-SeT displayed a somewhat lower overall specificity than CATT/*T. evansi* (94.8% *versus* 98.3%, χ^2 =13.37, p<0.001) but a considerably higher sensitivity (98.1% *versus* 84.4%, χ^2 =33.39, p<0.001). We conclude that the Surra Sero *K*-SeT may become an alternative for the CATT/*T. evansi* for sensitive detection of antibodies against *T. evansi* in domestic animals.

2. Introduction

Parasitological techniques such as Giemsa stained thin smears or thick drops and the microhaematocrit centrifugation technique (mHCT), are commonly used for the diagnosis of surra but their sensitivity is low due to the fluctuating and often low parasitaemia, particularly during the chronic stage of the disease (Büscher 2014). Therefore, serodiagnosis based on the detection of *T. evansi* specific antibodies is recommended by the World Organisation for Animal Health (Organisation Internationale des Epizooties, OIE) (OIE 2012). Within the mammalian host, the cell membrane of the trypanosome is covered by a monolayer of variant surface glycoprotein (VSG). This VSG coat is highly immunogenic and induces a strong antibody response in the host. As a result, trypanosomes that are recognised by VSG-specific antibodies are destroyed (Pays *et al.* 2004; Horn 2014). The VSG conferring the variant antigen type RoTat 1.2 is shared among most *T. evansi* strains, except in some rare *T. evansi* strains isolated from dromedary camels in Kenya (Verloo *et al.* 2001; Ngaira *et al.* 2005) and Ethiopia (Chapters 3 and 4). Several antibody detection tests have been developed that are based on the native VSG RoTat 1.2 including the Card Agglutination Test for Trypanosomosis (CATT/*T. evansi*), enzyme linked immunosorbent assay (ELISA/*T. evansi*) and immune trypanolysis (TL) (Bajyana Songa & Hamers 1988; Verloo *et al.* 1998; Lejon *et al.* 2005). To avoid the use of laboratory rodents for the production of native VSG RoTat 1.2, the N-terminal domain of VSG RoTat 1.2 has been expressed as recombinant protein in *Spodoptera frugiperda* insect cells and in *Pichia pastoris* yeast cells and used as antigen in ELISA and in latex agglutination (Urakawa *et al.* 2001; Lejon *et al.* 2005; Rogé *et al.* 2014). None of the above mentioned serological test formats complies with the ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid, equipment-free and delivered) (Mabey *et al.* 2004). Therefore, the development of an ASSURED serodiagnostic test for surra remains necessary (Büscher 2014).

Following the development of the HAT Sero *K*-SeT for sleeping sickness caused by *T. brucei gambiense*, we developed a similar test for the serodiagnosis of infection with *T. evansi*, the Surra Sero *K*-SeT (Büscher *et al.* 2013; Büscher *et al.* 2014). This test (Coris BioConcept, Gembloux, Belgium) is a lateral flow immunochromatographic test (ICT) for detection of RoTat 1.2- specific antibodies in blood, serum or plasma of any mammalian species. The test uses a fragment of VSG RoTat 1.2, produced as recombinant antigen in *Pichia pastoris* (Rogé *et al.* 2013). In the present study, we report on the diagnostic accuracy of the Surra Sero *K*-SeT on sera from various domestic animal species from different parts of the world in comparison with CATT/*T. evansi* and TL as reference test.

3. Materials and methods

Ethics statement

For the *in vivo* culture of trypanosomes in mice, ethical approval was obtained from the Veterinary Ethics Committee of the Institute of Tropical Medicine, Antwerp,Belgium under protocol BM2013-7.

Sera

A total of 806 sera from dromedary camels, water buffaloes, bovines, sheep, horses, dogs and alpacas were analysed (Table 5.1). These sera belong to the archived collection from the Institute of Tropical Medicine, Antwerp, Belgium.

Table 5.1: Host species, origin, year of collection, number and status in immune trypanolysis (TL) of all serum samples used in this study. TL pos: immune trypanolysis positive; TL neg: immune trypanolysis negative.

Species	Origin	Year	Number	TL pos	TL neg	Reference
Camel	Morocco	1997-1998	100	64	36	(Atarhouch *et al.* 2003)
Camel	Mali	1989-1991	97	34	63	(Diall *et al.* 1994)
Camel	Niger	1995	77	70	7	(Verloo *et al.* 1998)
Camel	Spain (Gran Canaria)	1997-1999	26	13	13	(Gutiérrez *et al.* 2000)
Water buffalo	Philippines	1995	100	82	18	**
Bovine	Suriname	1992-1993	82	0	82	**
Sheep	France	2007	81	0	81	(Desquesnes *et al.* 2008)
Sheep*	UK (Scotland)	1994	7	7	0	(Onah *et al.* 1996)
Horse	Diverse	2010-2012	50	0	50	**
Horse	Spain (Gran Canaria)	2009	50	0	50	(Gutiérrez *et al.* 2010)
Dog	Diverse	2013	74	0	74	**
Dog	Belgium	1988	25	0	25	**
Alpaca	The Netherlands	2012-2014	19	0	19	**
Alpaca	UK	2012-2014	18	0	18	**
Total			**806**	**270**	**536**	

* Experimentally infected

** Left-over specimens from reference diagnostic activities within the framework of the OIE Reference Laboratory for Surra at the Institute of Tropical Medicine, Antwerp, Belgium

Immune trypanolysis

TL was used as reference test for *T. evansi* specific antibodies since it is considered 100 % specific and has a high analytical sensitivity (Verloo et al., 2000). TL makes use of a cloned *T. evansi* population of live trypanosomes all expressing the VSG RoTat 1.2. In the presence of anti-RoTat 1.2 antibodies and of guinea pig complement, these trypanosomes will be killed by antibody-mediated complement lysis (Verloo *et al.* 2000; Van Meirvenne *et al.* 1995). TL was performed with a suspension of about 10^7 trypanosomes per milliliter as estimated by the Matching Method (Herbert and Lumsden, 1976). This suspension was freshly prepared by diluting the blood from a mouse infected with *T. evansi* RoTat 1.2 with guinea pig serum (GPS, Harlan, Horst, The Netherlands). Twenty five µl of the test serum were mixed with 25 µl of GPS in wells of U-bottom polystyrene microtitre plates (Sterilin, Newport, UK) and incubated at ambient temperature for 30 min. To this mixture, 50 µl of the trypanosome suspension were added. After 90 min at ambient temperature, antibody mediated lysis was assessed by phase-contrast microscopy at 25x10 magnification. When 50% or more of the trypanosomes were lysed, the specimen was considered positive for the presence of anti-RoTat 1.2 antibodies, indicating current or past infection with *T. evansi*.

CATT/*T. evansi*

CATT/*T. evansi* was used as one of the index tests. It is one of the OIE recommended tests for *T. evansi*-specific antibody detection CATT/*T. evansi* was carried out according to the instructions of the manufacturer with serum diluted 1:4 in CATT diluent.

Surra Sero K-SeT

Surra Sero *K*-SeT was the second index test of which the diagnostic accuracy was assessed in this study. The antigen in the Surra Sero *K*-SeT consists of recombinant rVSG RoTat 1.2, produced in *Pichia pastoris* (Rogé *et al.* 2013). Surra Sero *K*-SeT was performed according to the instructions of the manufacturer. Briefly, 15 µl of serum was dispensed in the sample application window of the cassette, followed by 85 µl of the migration buffer. After 15 min, the test result was read as positive if both the control and the test line were visible (even if very faint), negative if only the control line was visible and invalid if the control line was not visible (Figure 5.1).

Statistical analysis

All data were recorded in Microsoft Excel (Microsoft, Version 2010). Sensitivities and specificities with 95% confidence intervals (CI) were calculated using STATA /MP 13.1 (StataCorp. 2013) with TL as reference test. McNemar χ^2 was calculated to test differences in sensitivity and specificity between the two index tests, Surra Sero *K*-SeT and CATT/*T. evansi*. The level of agreement between the diagnostic tests was determined using Cohen's kappa coefficient (Landis & Koch 1977; Viera & Garrett 2005). Probability (p) values < 0.05 were considered as significant.

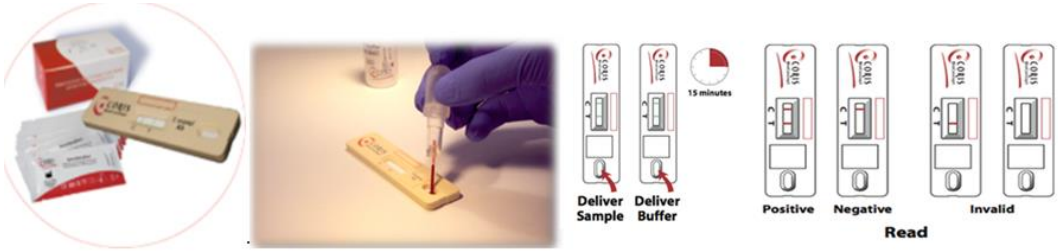

Figure 5.1: Surra Sero *K*-SeT Kit. Fifteen µl of blood or plasma are applied in the specimen window, followed by 85 µl of chase buffer. After 15 min, the reaction is read as positive (red test line and red control line), negative (red control line only) or invalid (no visible control line).

4. Results

The results obtained with the 806 sera and the three diagnostic tests are represented in a contingency table (Table 5.2). All diagnostic tests showed almost perfect agreement (κ > 0.8). The test agreement was highest between Surra Sero *K*-SeT and TL (κ=0.91, 95% CI 0.841-0.979) and somewhat lower between CATT/*T. evansi* and TL (κ=0.85, 95% CI 0.785-0.922) and between CATT/*T. evansi* and Surra Sero *K*-SeT (κ=0.81, 95% CI 0.742-0.878).

Table 5.2: Contingency table with results of all sera tested in the immune trypanolysis (TL), the Card Agglutination Test for *T. evansi* Trypanosomosis (CATT/*T. evansi*) and the Surra Sero *K*-SeT.

	CATT		**Sero *K*-SeT**			**Sero *K*-SeT**	
TL	pos	neg	pos	neg	CATT	pos	neg
pos	228	42	265	5	pos	231	6
neg	9	527	28	508	neg	62	507

The sensitivities and specificities of CATT/*T. evansi* and Surra Sero *K*-SeT using TL as the reference test, both overall and for each host species, are shown in Table5. 3. Since all sera from bovines, horses, dogs and alpacas were negative in TL, sensitivities of the index tests could not be calculated. Overall, as well as separately for camels and water buffaloes, the sensitivity of the Surra Sero *K*-SeT was significantly higher ($p < 0.001$) than the sensitivity of the CATT/*T. evansi* (Table 5.3). All seven experimentally infected sheep sera tested positive in both index tests. On the other hand, the overall specificity of Surra Sero *K*-SeT was significantly lower than that of CATT/*T. evansi* ($p < 0.05$) as was the specificity of Surra Sero *K*-SeT for bovines, dogs and, in particular, alpacas. Of this latter species, 6 out of 37 sera, all from UK, were false positive in the

Surra Sero *K*-SeT while all were negative in CATT/*T. evansi*. Of all 806 sera tested, four bovines and one sheep were false positive in both CATT/*T. evansi* and Surra Sero *K*-SeT. No invalid results were obtained with Surra Sero *K*-SeT.

Table 5.3: Sensitivity (Se) and specificity (Sp) with 95% confidence interval (CI) of the Card Agglutination Test for *T. evansi* Trypanosomosis (CATT/*T. evansi*) and the Surra Sero *K*-SeT according to the host species and with immune trypanolysis (TL) as reference test. na = not applicable, p = probability.

Host	**Sensitivity (95% CI)**			**Specificity (95% CI)**		
species	**CATT/*T. evansi***	**Surra Sero *K*-SeT**	**Chi square, p**	**CATT/*T. evansi***	**Surra Sero *K*-SeT**	**Chi square, p**
Camel	87.3	97.8	15.70	99.2	95.8	2.67
	(81.7-91.4)	(94.5-99.1)	< 0.001*	(95.4-99.9%)	(90.5-98.2)	0.103
Water	76.8	98.8	18.00	100	94.4	1.00
buffalo	(66.6-84.6)	(93.4-99.8)	< 0.001*	(82.4-100)	(74.3-99.0)	0.317
Bovine	na	na	na	95.1	89.0	5.00
				(88.0-98.7)	(80.4-94.1)	0.025*
Sheep	100	100	na	96.3	96.3	0.00
	(64.6-100)	(64.6-100)		(89.7-98.7)	(89.7-98.7)	1.000
Horse	na	na	na	99.0	100	1.00
				(94.6-99.8)	(96.3-100)	0.317
Dog	na	na	na	100	96.0	4.00
				(96.3-100)	(90.0-98.4)	0.046*
Alpaca	na	na	na	100	83.8	6.00
				(90.6-100)	(68.9-92.4)	0.014*
Overall	84.4	98.1	33.39	98.3	94.8	13.37
	(79.6-88.3)	(95.7-99.2)	< 0.001*	(96.8-99.1)	(92.6-96.4)	< 0.001*

* statistically significant

5. Discussion

In this study, we evaluated the diagnostic accuracy of Surra Sero *K*-SeT, a newly developed immunochromatographic serodiagnostic test, in comparison with CATT/*T. evansi*, the latter being one of the antibody detection tests recommended by OIE (OIE 2012). Cohen's kappa analysis showed an almost perfect agreement between both tests ($\kappa > 0.8$). Surra Sero *K*-SeT was significantly more sensitive than CATT/*T. evansi* but its specificity was lower, particularly in bovines and alpacas. The higher sensitivity may be explained by the fact that undiluted serum is tested in the Surra Sero *K*-SeT while in the CATT/*T. evansi*, the test serum is diluted (1:4). The lower specificity in bovines may be explained by the detection of antibodies generated by other infections that cross react with the recombinant RoTat 1.2 VSG. For example, the bovine sera originated from Suriname where, at the time of collection, no *T. evansi* was reported but where

false positive reactions in CATT/*T. evansi* were attributed to the presence of *T. vivax* (Van Vlaenderen 1996; Dávila & Silva 2000). Similar cross reactions caused by *T. vivax* and *T. cruzi* infections were previously observed in an ELISA where the antigen consisted of a crude lysate of *T. evansi* trypomastigotes (Desquesnes *et al.* 2007). Although not tested in this study, we do not expect the recombinant RoTat 1.2 antigen to cross react with antibodies raised against non-pathogenic trypanosomes such as *T. theileri* or *T. melophagium* since in that case, the specificity of the Surra Sero *K*-SeT in bovine, buffalo and sheep would be much lower. The 16% alpaca sera that were false positive in Surra Sero *K*-SeT while true negative in CATT/*T. evansi* are more puzzling since all the animals originated from a country that is non-endemic for pathogenic trypanosomes (UK). As a consequence, we cannot recommend Surra Sero *K*-SeT for testing of alpaca. It would be interesting to investigate whether the sub-optimal specificity of the Surra Sero *K*-SeT can be overcome by the use of alternative recombinant VSG RoTat 1.2 expressed in *Escherichia coli* or in *Spodoptera frugiperda* (Sengupta *et al.* 2012; Urakawa *et al.* 2001).

We were not able to include sera from animals infected with *T. evansi* type B, which we consider a limitation of our study. Although Surra Sero *K*-SeT showed high sensitivity when tested on different host species in the current study, it may not detect infections with *T. evansi* type B, known not to express RoTat 1.2 VSG (Ngaira *et al.* 2005; Ngaira *et al.* 2003; Ngaira *et al.* 2004; Njiru *et al.* 2004). This rare trypanosome type was isolated for the first time from camels in Kenya and may also occur in Sudan and Ethiopia (Borst *et al.* 1987; Hagos *et al.* 2009; Salim *et al.* 2011). Therefore, it might be of interest to combine different antigens in the Surra Sero *K*-SeT, thus broadening the spectrum of antibodies that can be detected, including antibodies detectable in early *T. evansi* type A infections before the apparition of anti-RoTat 1.2 antibodies. Examples of antigens that might be combined with the recombinant RoTat 1.2 VSG in the Surra Sero *K*-SeT are recombinantly expressed fragments of invariant surface glycoprotein 75 (ISG75) and tandem repeat cytoskeleton protein GM6 that have been shown to bear diagnostic potential in camel, goat and water buffalo (Tran *et al.* 2009; Rogé *et al.* 2013; Nguyen *et al.* 2012; Müller *et al.* 1992). Of particular interest is GM6 of which a 4 repeat fragment derived from *T. evansi* (TeGM6-4r) has been expressed in *E. coli* and incorporated in a lateral flow ICT (Nguyen *et al.* 2015). When tested on naturally infected sheep, goat and cattle in KwaZulu-Natal, South Africa, it showed moderate diagnostic potential (Nguyen *et al.* 2015) that may be exploited in combination with the recombinant RoTat 1.2 VSG used in the Surra Sero *K*-SeT. Increasing the sensitivity of a diagnostic test for surra, even with the risk of decreasing its specificity is particularly of interest in situations where it is important to detect all infected animals. For instance when moving susceptible animals from an endemic to a non-endemic country or when disease control measures include the identification and treatment of reservoir hosts.

As mentioned earlier, ICTs have several advantages over other test formats such as ELISA, TL or card agglutination tests. ICTs come as individually packed tests, can be performed with whole blood, are equipment-free and thermostable and therefore are perfectly fit for use in the field or in a rudimentary laboratory environment. Thus, the Surra Sero *K*-SeT is applicable in rural

veterinary clinics where herdsmen come with their animals for vaccination, acaricide spraying and treatment for common diseases. Also, laboratories where diagnosis of surra is performed irregularly and on a small number of animals, may benefit from the individual test format of the Surra Sero *K*-SeT. Taken together, the management of surra, that till today is primarily based on treatment of clinical cases only, may drastically change when rapid diagnostic tests, like the Surra Sero *K*-SeT, become available.

We conclude that the Surra Sero *K*-SeT may become an ASSURED alternative for the CATT/*T. evansi* for sensitive detection of antibodies against *T. evansi* in domestic animals depending on the epidemiological situation. In regions where the prevalence is high, like we observed in the epdimiological part of our study, the higher sensitivity of the Surra Sero *K*-SeT compared to CATT/*T. evansi* (if confirmed with whole blood in stead of plasma), is advantageous since the Surra Sero *K*-SeT will have a higher negative predictive value, even when the specificity is lower than of CATT/*T. evansi*. On the other hand, in regions where the prevalence is low, it would be more efficient to use the CATT/*T. evansi* that is more specific, since its positive predictive value will be higher than of the Surra *K*-SeT. When prevalences are close to zero, a diagnostic test with a specificity near 100% is needed to reach an acceptably high positive predictive value. In that case, neither Surra Sero *K*-SeT nor CATT/*T. evansi* fulfil the requirements and rather should immune trypanolysis be preferred to screen animals in a population at risk or to confirm a positive result obtained with CATT/*T. evansi* or Surra Sero *K*-SeT. When it is not possible to conduct immune trypanolysis, an ELISA/*T. evansi* adjusted for high specificity by applying a high cut-off O.D. may be a good alternative (Verloo *et al.* 2000). It is clear that further research is necessary to increase the specificity of the test and to assess its diagnostic accuracy when applied on whole blood under field conditions.

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General discussion

Animal African trypanosomosis, with an estimated annual loss of up to 4.5 billion USD is still one of the major parasitic disease constraints to animals' productivity in sub-Sahara Africa, while human African trypanosomosis has reached the point where elimination is being discussed (Auty *et al.* 2015; Morrison *et al.* 2016; Holmes 2014; Holmes 2014; Shaw *et al.* 2014). The impact of AAT on local communities is the result of complex interactions between environmental, political, socio-cultural, entomological and livestock management factors (Bouyer *et al.* 2013). In recognition of the need for coordinated actions against AAT, the Pan-African Tsetse and Trypanosome Eradication Campaign (PATTEC), was established in 2000. However, many of the communities afflicted by AAT have insufficient resources available for its control and are not always reached by control programmes (Holt *et al.* 2016). Moreover, PATTEC has hitherto paid little attention to NTTATs, caused by *T. evansi* (and *T. vivax*), despite the serious economic losses due to surra in camel herding pastoral area of Africa.

For example, in Somaliland, a country with a gross domestic product of 347 USD per citizen in 2014, the annual loss of revenue due to surra in camels was estimated 223.1 million USD of which the majority was from decreased milk production and from body condition loss. The benefit of controlling *T. evansi* infection in camels was estimated 720 USD, and thus higher than the gross domestic product per citizen per year (Salah *et al.* 2015; World Bank 2014). For Indonesia, Peternakan estimated that the annual loss from morbidity and mortality in bovine and buffaly was 28 million USD (Payne *et al.* 1994b; Reid 2002). The estimated annual cost of *T. evansi* to the Pantanal region's ranchers was about 2.4 million USD owing to mortality of 6462 horses peryear (Seidl *et al.* 1998). These examples show that the global impact of surra may well be above the impact of TTAT that is confined to Africa.

In Ethiopia, compared to TTAT, limited attention is given to control of NTTAT (Sinshaw *et al.* 2006; Fikru *et al.* 2012). Studies on animal trypanosomosis in non-tsetse areas of Ethiopia, are limited, fragmented and mostly making use of poorly sensitive and specific diagnostic tests, thus inevitably underestimating or overestimating the impact of NTTAT on livestock production in the country. *T. evansi* strains from Ethiopia were typically lacking in the cryobank of the OIE Reference Centre for surra at ITM and elsewhere. Furthermore, there were no published reports on drug sensitivity profiles of *T. evansi* strains from Ethiopia. This PhD study was initiated 1° to conduct, for the first time, a large scale epidemiological survey on *T. evansi* and *T. vivax* in Northern Ethiopia using the most advanced parasitological, serological and molecular techniques, 2° to isolate and characterise Ethiopian *T. evansi* strains and 3° to make improvements on the molecular and serological diagnosis of surra.

Epidemiology of NTTAT in Northern Ethiopia

In the epidemiological study, we used the mHCT and CATT/*T. evansi,* respectively the OIE recommended parasitological and serological tests, for screening the animals (Figure 6.1) (OIE 2012; OIE 2013b). Collected blood samples were subsequently processed in species- and type-specific PCRs for *T. vivax, T. evansi* type A and *T. evansi* type B and in TL, the OIE recommended gold standard antibody detection test for serdiagnosis of surra (OIE 2012; Njiru *et al.* 2006; Claes *et al.* 2004; Fikru *et al.* 2014).

Figure 6.1: Field activities: registering of animals, blood sample collection, separation of blood components and mHCT.

Among the 754 camels, we observed parasites in 4% of the animals and *T. evansi* specific antibodies in 10.5%. Also the molecular diagnostics showed an important fraction of the animals infected with *T. evansi* type A (11.7%), *T. evansi* type B (0.5%) and even *T. vivax* (3.4%). These high prevalences of NTTAT in camel correspond with findings of other recent studies on Ethiopian camels conducted in Afar and Oromia (Fikru *et al.* 2015; Hagos *et al.* 2009). Interestingly, we confirmed the presence of *T. evansi* type B in Ethiopia, at least in Afar. Indirect evidence of non-RoTat 1.2 expressing trypanosomes circulating in Ethiopian camels was provided in a previous study conducted in Oromia (Hagos *et al.* 2009). Our data, together with the indirect evidence of *T. evansi* type B circulating in Chad and in Sudan, indicate that *T. evansi* type B is probably present in more East African countries, beyond Kenya where it was first discovered (Ngaira *et al.* 2005; Ngaira *et al.* 2004; Njiru *et al.* 2006; Sánchez *et al.* 2015; Boid 1988; Salim *et al.* 2011). Whether this parasite has reached Latin America or Asia remains unanswered. In a recent survey on camels in the Cholistan Desert of Pakistan *T. evansi* type B was not observed although *T. evansi* type A was highly prevalent (30%) (Tehseen *et al.* 2015). Our study revealed, for the first time, mixed infections with *T. evansi* type A and *T. evansi* type B. Whether both types cause differential pathology has not been studied so far. We noted an average PCV of 26% in *T. evansi*

type A infected camels and 22% in type B infected animals but the numbers are too small to draw firm conclusions on these data. So far, only camels have been shown to harbour type B despite epidemiological studies on equines, cattle and small ruminants in Kenya and Ethiopia (Njiru *et al.* 2006; Birhanu *et al.* 2015a). Therefore, one may suggest that *T. evansi* type B may be restricted to camels. Experimental infections of various domestic animal species with *T. evansi* type B could give a better understanding on the host pathogen interaction of this parasite. The finding of camels infected with *T. vivax* in our study corresponds with the data presented by Fikru and colleagues (Fikru *et al.* 2015). Pathogenicity of *T. vivax* in camels has not been studied in detail. In our survey *T. vivax* infection was accompanied by a low PCV (24%). The mobility of camels either in search of food and water or for pack transport puts them at risk of attracting also tsetse-transmitted trypanosomes and making them unintentional vehicles of these trypanosomes into non adjacent tsetse infested zones across tsetse free areas. Indeed, infection of camels with *T. brucei* and *T. congolense* has been documented in Kenya and Somalia but was not observed in our study (Dirie *et al.* 1989; Wilson *et al.* 1983).

There is an encouraging trend of research in mechanically transmitted *T. vivax* in cattle from Ethiopia (Sinshaw *et al.* 2006; Fikru *et al.* 2012; Dagnachew *et al.* 2015a; Cherenet *et al.* 2006). In our survey, 493 cattle were included of which 7% were parasitologically positive and 37% were serologically positive in CATT/*T. evansi*. Presence of *T. evansi* and *T. vivax* was confirmed with RoTat 1.2 PCR (6%) and TvPRAC PCR (3%). However, among the parasitologically confirmed cattle, only three and one were positive in TvPRAC PCR and RoTat 1.2 PCRs respectively. Taking into account the low analytical sensitivity of TvPRAC PCR, caused by its single copy target sequence, the real prevalence of *T. vivax* in cattle may be higher (Fikru *et al.* 2014). Also for the RoTat 1.2 PCR it is known that its analytical sensitivity is lower than of a PCR targeting multicopy sequences, like ITS1 PCR. On the other hand, ITS1 PCR is poorly sensitive for *T. vivax* caused by the high GC content of its DNA. Also, ITS1 PCR is less specific and can generate many aspecific amplicons, particularly in bovine samples (Fikru *et al.* 2016). The ITS1 PCR results on the parasitologically positive cattle revealed 4 *T. vivax* that were not detected with TvPRAC PCR*,* 2 *T. theileri,* 8 negatives and 18 with amplicons of different lengths that could not be interpreted correctly. Due to the complexity of the aspecific amplicon profiles, we didn't undertake their sequencing.

Among 445 small ruminants, we observed 0.4% parasitologically positive animals and 13% seropositives for antibodies against *T. evansi* type A. In PCR, about 3% were positive for *T. evansi* type A and for *T. vivax*. These prevalences are lower than in camels and cattle. Yet, the fact that they can be infected by all pathogenic trypanosomes, that they usually exhibit a mild or asymptomatic form of the disease and that they are rather neglected in trypanosomoses control projects make them potentially important reservoirs of NTTAT (Gutiérrez *et al.* 2006b; Ngeranwa *et al.* 1991; Ngeranwa *et al.* 1993).

A total of 119 equines (horses, mules and donkeys) were included in our survey. All were negative in the mHCT and antibodies against *T. evansi* type A were only detected in donkeys although PCR revealed *T. evansi* DNA in high proportions of the horses (28%), mules (10%) and donkeys (6%). Only donkeys were positive in *T. vivax* specific PCR (3.6%). Taken together, NTTAT is present in equines in Tigray and Afar although parasitaemia seems to remain under the detection limit of mHCT. Similar observations were made in a survey on horses in the Arsi Bale highlands antibodies were detected in 28%, 25% and 19% of 646 horses with respectively the CATT/*T. evansi*, LATEX/*T. evansi* and ELISA/*T. evansi* without any positive in Giemsa stained thick drop and in mHCT (Hagos *et al.* 2010a). The usual subclinical form of the disease and the transient and low parasitaemia, limiting the detection of circulating *T. evansi* parasites in blood has been reported by others (Berlin *et al.* 2009; Berlin *et al.* 2010; Pascucci *et al.* 2013). However, surra in horses can be a very serious diseases with up to 50% mortality as reported after an outbreak in the Pantanal in the Brazilian Mato Grosso state (Silva *et al.* 1995). A study conducted on 237 horses in the Arsi-Bale highlands of Ethiopia revealed parasites in 4.6% of the animals (with mHCT) and very high percentages of serologically (37% in CATT/*T. evansi*) and molecularly positive animals (up to 48% in 18S PCR) (Gari *et al.* 2010). Another study, conducted by Hagos and colleagues in 880 horses from Bale highlands in Oromia region also found a seroprevalence of 20% CATT/*T. evansi* (Hagos *et al.* 2010b). The differentiation in horses between infection with *T. evansi* and the sexually transmitted *T. equiperdum* remains challenging and is only possible on the basis of RoTat 1.2 specific antibodies and on the absence of maxicircles in the kDNA of *T. evansi*. *T. equiperdum* can be revealed by maxicircle specific PCR (Claes *et al.* 2003b; Claes *et al.* 2004; OIE 2013a; Li *et al.* 2007a). Thus, together with the other studies on equine NTTAT, our survey indicates that surra is important in equines in Ethiopia.

Due to the persistence of trypanosome-specific antibodies in cured animals and to reactions with antibodies against non-specific epitopes, a serological test like the CATT/*T. evansi* might overestimate the prevalence of *T. evansi* (Verloo *et al.* 2000; Büscher 2014; Urakawa *et al.* 2001; Hilali *et al.* 2004). In Ethiopia, where both *T. evansi* and *T. vivax* are endemic, cross reactivity in CATT/*T. evansi* is unavoidable but should not be problematic since current treatment for AAT is not species-specific (Büscher 2001; Uzcanga *et al.* 2002; Camargo *et al.* 2004). In Somaliland, biannual treatment of all seropositive camels was found the most efficient control strategy against surra (Salah *et al.* 2015). To discriminate between VSG-specific and non-specific reactions in CATT/*T. evansi*, sera or plasma can be tested in TL which is 100% VAT specific and recommended by OIE as the gold standard for serodiagnosis of surra due to *T. evansi* type A. The test is available only at the OIE Reference Laboratory for Surra at ITM. Due to sanitary and phytosanitary regulations, we could not bring animal plasma into Belgium. Hence, plasma samples collected during the survey were dried on Whatman 4 filter paper and the TL was performed on the antibodies eluted from the filter paper. Unexpectedly, only 34 animals were positive in TL resulting in poor agreement between CATT/*T. evansi* and TL. We have no firm explanation why TL was negative in all RoTat 1.2 PCR positive horses and cattle. A limited loss in

sensitivity of TL when performed on filter paper eluates compared to plasma has been documented (Holland *et al.* 2002). Recently, we have observed that drying 500 µl of plasma on the cotton plug of a Salivette, a device that is designed for the hygienic collection of saliva (Sarstedt, Germany), considerably improves the yield of antibodies that can be eluted, hence increasing the analytical sensitivity of TL on eluted antibodies (Figure 6.2).

On the other hand, while TL detects exclusively variant specific antibodies, CATT/*T. evansi* detects also antibodies directed against non-variant epitopes of VSG RoTat 1.2 and other surface exposed antigens. Thus, infection with other trypanosomes, e.g. *T. vivax*, may lead to a positive result in CATT/*T. evansi* (Van Vlaenderen 1996; Uzcanga *et al.* 2004; Büscher 2001). This cross-reactivity caused by *T. vivax* infection may explain why CATT/*T. evansi* positive cattle specimens remained negative in TL but had been in contact with *T. vivax* only. Parasitological and molecular tests are highly specific and provide conclusive evidence for ongoing infection, however their sensitivity is affected because of the often low number of circulating parasites. For diagnosis of AAT, parasite concentration techniques such as the mHCT that was used in this study are highly recommended (Büscher 2014).

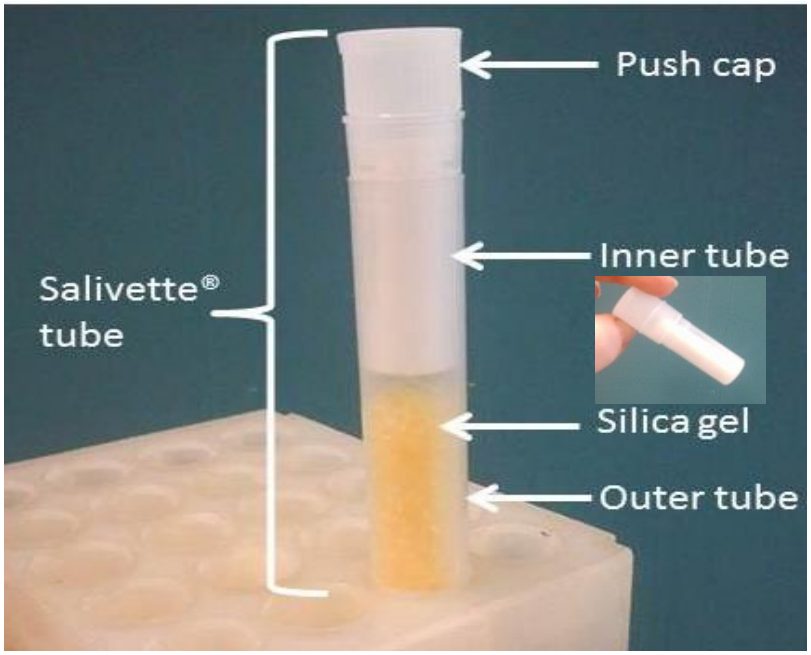

Figure 6.2: Salivette tube with push cap, inner tube, specimen absorbing plug and silica gel.

Isolation and genotypic characterization of *T. evansi*

For the isolation of new trypanosome strains from Ethiopia, a total of 68 parasitologically positive buffy coat samples from cattle (36), dromedary camels (30), goat (1) and sheep (1) were inoculated in Swiss albino mice. In parallel, the buffy coat specimens were characterized by species specific PCRs. We achieved a high isolation success rate of 32% but only from camels and only *T. evansi,* confirming the appropriateness of mice to isolate *T. evansi* (Mekata *et al.* 2013). Most importantly, we isolated two Ethiopian *T. evansi* type B stocks, decades after its first isolation in Kenya. Not surprisingly, we were unable to isolate *T. vivax*. This species is notoriously difficult to adapt in laboratory rodents and so far, only one mouse-adapted Nigerian *T. vivax* strain is available for research (Guerreiro *et al.* 2005; Gardiner 1989). Isolation of *T. vivax* failed in immunosuppressed young zebu calves and in goats and only a short parasitaemic period was observed where after the parasites remained undetectable in the blood. Only in immunosuppressed Friesian Holstein calves, sufficiently high parasitaemia could be obtained to prepare cryostabilates and to purify the parasites for molecular characterization (Fikru 2015). In Brazil and Venezuela, local *T. vivax* strains from cattle were adapted to immunosuppressed sheep with subsequent purification of the parasites from the blood by Percoll gradient centrifugation (González *et al.* 2005; Greif *et al.* 2013).

As expected, all 14 *T. evansi* stocks isolated in this study were negative for all four maxicircle genes (ND4, ND5, ND7, A6) (Borst *et al.* 1987; Dean *et al.* 2013; Domingo *et al.* 2003). All stocks that contain RoTat 1.2 VSG, except the akinetoplastic MCAM/ET/2013/MU/09, were positive in minicircle A PCR, both in their pellet DNA and buffy coat DNA (Njiru *et al.* 2006). The presence of a kinetoplast in the other 13 stocks was demonstrated using DAPI staining and fluorescence microscopy. The occurrence of naturally akinetoplastic *T. evansi* strains has been documented in Latin America and China (Stevens *et al.* 1989; Ventura *et al.* 2000; Ou *et al.* 1991; Lun & Vickerman 1991). We used the minisatellite locus MORF2-REP to confirm that the putative mixed *T. evansi* type A and B stocks were genuine mixed infections and not hybrids. From this analysis, it appeared that our Ethiopian *T. evansi* type A stocks are different from the classical *T. evansi* type A RoTat 1.2, isolated in 1980 from water buffalo in Indonesia. Also the two Ethiopian *T. evansi* type B stocks are different from the classical *T. evansi* type B KETRI 2479. These findings underline the interest of the minisatellite locus MORF2-REP to analyse diversity among *T. evansi* strains, even at type level (Biteau *et al.* 2000).

During our study, we were confronted with the limited reliability of molecular tests for typing *T. evansi* strains. The RoTat 1.2 VSG gene is useful as a marker of *T. evansi* type A but the RoTat 1.2 PCR developed by Claes and colleagues that generates a short amplicon (205 bp) unexpectedly was positive on DNA of *T.b. gambiense* LiTat 1.R, *T.b. brucei* AnTat 1.1 and *T. evansi* KETRI 2479 when high amount of target DNA is used in the PCR (Claes *et al.* 2004). Therefore, we recommend the use of the RoTat 1.2 PCR from Urakawa and colleagues that consistently generates a 488 bp fragment only in *T. evansi* type A (Urakawa *et al.* 2001). Despite previous

work that proposed the JN 2118Hu VSG gene as a specific marker for *T. evansi* type B (Ngaira *et al.* 2005), we cannot recommend its use. Indeed, we observed that the JN 2118Hu specific PCR cross reacts with *T.b. brucei* AnTat 1.1 and *T.b. gambiense* type II ABBA underlining the importance of including sufficiently diverse trypanosomes representing species, subspecies and type for the evaluation of new molecular typing markers. Based on the recent work of Carnes *et al.* who used SNPs in the gene of the F1-ATP synthase γ subunit for genotyping of dyskinetoplastic *Trypanozoon* (Carnes *et al.* 2015), we cloned and sequenced the full gene of F1-ATP synthase γ subunit of several *T. evansi* and other closely related *Trypanozoon* strains. Interestingly, all Ethiopian *T. evansi* type A had a polymorphism corresponding to the classical type A group. The Ethiopian *T. evansi* type B had a similar profile as KETRI 2479 and mixed infections showed a mixed polymorphism profile. In this regard, we confirmed that polymorphism within the F1-ATP synthase γ subunit gene might become an interesting target for genotyping of *T. evansi* stocks, and is not related to a VSG repertoire that can change over time, nor to the presence of a kinetoplast that can be lost during evolution. We therefore recommend the development and evaluation of an allele specific F1-ATP synthase γ subunit PCR for reliable typing of *T. evansi* stocks during epidemiological surveys.

In vitro adaptation and drug sensitivity profiling of Ethiopian *T. evansi* stocks

The adaptation of the Ethiopian *T. evansi* stocks to *in vitro* culture with HMI-9 HS medium was successful in 5 of the 14 stocks. Unexpectedly, mixed type A and type B populations that could be propagated *in vivo* in mice became exclusively type B when adapted to *in vitro* culture underlining the possible selection bias of the method used for isolation of trypanosomes. This phenomenon necessitates regular checking of the *T. evansi* type during isolation and adaptation experiments. The *in vitro* drug sensitivity testing on some of the Ethiopian *T. evansi* stocks indicated no drug resistance against melarsomine dihydrochloride, suramin, isometamidium hydrochloride and diminazene diaceturate. However, in a parallel MSc study following the standard protocol of Eisler and coworkers, we observe that the same stocks are also sensitive to 20 mg/kg diminazene diaceturate *in vivo* but appear resistant to 1 mg/kg isometamidium hydrochloride *in vivo* (Eisler *et al.* 2001). The discrepancy for sensitivity of *T. evansi* to isometamidium hydrochloride *in vivo* and *in vitro* has been documented and might be due to *in vivo* metabolisation of the drug to a less active form (Zhang *et al.* 1991). Although isometamidium hydrochloride is known to accumulate in the mitochondrion, *in vivo* testing with this drug in the akinetoplastic stock shows no trypanocidal activity in 100% of the infected mice thus corresponding with the recent finding that kinetoplast independent resistance of *T. evansi* type A to isometamidium hydrochloride is associated with the A281 deletion in the ATP F1 γ subunit gene although other mechanisms may be involved as well (Gould & Schnaufer 2014; Dean *et al.* 2013; Baker *et al.* 2015). The *in vivo* assays further show that low doses of melarsomine dihydrochloride (0.125 mg/kg), as used by Gillingwater and coworkers, are not sufficient to cure mice from infections with the Ethiopian *T. evansi* stocks (Gillingwater *et al.* 2010). Other studies report treatment failure with low doses of cymelarsan in buffaloes (0.25

mg/kg to 3 mg/kg), goats (0.3 mg/kg), mice (0.25 mg and 0.5 mg/kg) and cattle (0.5 mg/kg) (Lun *et al.* 1991; Payne *et al.* 1994a; Hagos *et al.* 2010c; Zweygarth *et al.* 1992; Syakalima *et al.* 1995). Therefore, the dosis in the *in vivo* experiment was increased to 2 mg/kg that showed 100% cure of mice infected with *T. equiperdum* (Hagos *et al.* 2010c). In the *in vivo* experiments in mice, the parasites are cleared in all mice at day 60 post treatment. All the *in vitro* adapted stocks (after 30 and 60 days) remained infective to mice which is in agreement with observations of Baltz and colleagues up to three months *in vitro* propagation (Baltz *et al.* 1985). Another study reported loss of rodent infectivity after 14 months of *in vitro* propagation (Zweygarth *et al.* 1990). In *in vitro* culture, a progressive loss of kinetoplasts was observed. It has been suggested that either a selective enrichment of dyskinetoplastic cells from an initial trypanosome population containing a few dyskinetoplastic organisms is taking place during *in vitro* propagation or that the *in vitro* mutations lead to the loss of kinetoplasts (Zweygarth *et al.* 1990).

Diagnostic accuracy of the Surra Sero *K*-SeT

The strong humoral immune response resulting from an infection with trypanosomes is exploited for serodiagnosis (Büscher 2014). Next to ELISA, IFAT and TL, all bound to well-equipped laboratories, the CATT/*T. evansi* is an OIE recommended antibody detecting serological test for serodiagnosis of surra (OIE 2012). The CATT/*T. evansi* can be performed in the field but is still dependent on electricity and equipment. In Human African Trypanosomosis (HAT), a similar test for mass screening in the field exists, the CATT/*T.b. gambiense*, produced as well at ITM, Antwerp but suffering from the same limitations as CATT/*T. evansi*. Together with Coris BioConcept, a Belgian diagnostic company, ITM has successfully developed the first real ASSURED RDT for HAT, the HAT Sero *K*-SeT (Büscher *et al.* 2013; Büscher *et al.* 2014). Following the success of the HAT Sero *K*-SeT, the Surra Sero *K*-SeT was developed by the same partners following the same principle, i.e. detection of VSG-specific antibodies in blood, plasma or serum. Our PhD study was the first to perform a large scale evaluation of the Surra Sero-*K*-SeT on sera from different domestic animal species. The overall sensitivity of Surra Sero *K*-SeT proved significantly higher but the specificity was significantly lower than that of CATT/*T. evansi*. This lower specificity was mainly due to an unexpected and hitherto unexplained low specificity of Surra Sero *K*-SeT in alpacas (Chapter 3, (Birhanu *et al.* 2015b)). High sensitivity of a serodiagnostic test for surra is of interest in situations where it is important to detect all infected animals, particularly when moving susceptible animals from an endemic to a non-endemic country or when disease control measures include the identification and treatment of reservoir hosts. Hence, the Surra Sero *K*-SeT may become an ASSURED alternative for the CATT/*T. evansi* for sensitive detection of antibodies against *T. evansi* in domestic animals except alpacas. On the other hand, since the Surra Sero *K*-SeT is designed to detect RoTat 1.2-specific antibodies, it is not expected to react with the blood from camels infected with *T. evansi* type B. Since this type may be more widely distributed in Eastern Africa than previously recognized, it is of interest to adapt the Sero *K*-SeT to detect also antibodies against this type of *T. evansi*. In the absence of known *T. evansi* type B specific

antigens, incorporation of invariant antigens such as ISG75, ISG65 and GM6 that are common to all *T. evansi* types and all *Trypanozoon* taxa could be explored. Also in HAT, where some *T.b. gambiense* types do not express the two VSGs used as antigen in the HAT Sero *K*-SeT, efforts are going on to include ISG65 as an alternative antigen (Sullivan *et al.* 2013; Rooney *et al.* 2015). Of particular interest is the cytoskeleton tandem repeat protein GM6, that has been expressed in *E. coli* and incorporated in a prototype ICT with good diagnostic potential in water buffalo, sheep, goats and cattle (Müller *et al.* 1992; Nguyen *et al.* 2015; Nguyen *et al.* 2014; Nguyen *et al.* 2012).

General conclusions and perspectives

In conclusion, this doctoral study showed that NTTAT due to *T. evansi* type A, *T. evansi* type B and *T. vivax* is an important threat to the health of domestic animals in Tigray and Afar regions of Northern Ethiopia. Molecular diagnostic tests prove that not only camels and bovine are affected but also equines and small ruminants underlining the necessity of considering these domestic animals when developing NTTAT control strategies. With a prevalence of 12% in camels, an animal that becomes more and more important under the present climate change challenges, the negative impact of *T. evansi* on the living condition of pastoral communities can hardly be overestimated. Unfortunately, NTTAT in Ethiopia are rather neglected. Control interventions are not coordinated, access to trypanocidal drugs and veterinary care in remote areas is limited and animal health workers lack the necessary skills and tools for proper diagnosis. This negligence is not confined to Ethiopia but is a general aspect of NTTAT. As an example, the Global Alliance for Livestock Veterinary Medicine (GALVmed), that is the only international organization working on diagnostics, drug and vaccines against *T. congolense* and *T. vivax,* has not yet incorporated AAT due to *T. evansi*, *T.b. brucei* and *T. equiperdum* in its portfolio (http://www.galvmed.org/en/news/new-drugs-fight-nagana/). Having confirmed the presence of *T. evansi* type B in Ethiopia, we believe that our study will inspire other researchers to further investigate the epidemiology of *T. evansi* type B worldwide. We are convinced that only RDTs that are accurate and cheap can be useful for diagnosis of AAT in poor resource veterinary clinics and that the current Surra Sero *K*-SeT must be further improved to allow serodiagnosis of *T. evansi* type B and, if possible, other pathogenic trypanosomes like *T. vivax*. Access to RDTs for NTTAT may also facilitate field studies on co-infections of trypanosomes with viral and bacterial pathogens in diverse domestic host species.

In view of further investigations on NTTAT, a number of limitations of this doctoral study has to be mentioned. For example, it was not possible to investigate all parameters that play a role in the epidemiology of NTTAT. We did not consider the effect of season, vector density, nutritional status of the animal and role of reservoir host. No detailed clinical examination of the study subjects, particularly camels infected with *T. evansi* type A and B was done. Comparison of the epidemiology of NTTAT between Tigray and Afar was not possible due to the significant differences in number of examined animal species per region and the number of sampled equines was too low to draw firm conclusions on NTTAT prevalence in these host species. Stained

blood smears, that could have allowed morphological distinction between *Trypanozoon*, *T. vivax*, *T. congolense* and *T. theileri* were not collected. Due to the sanitary and phytosanitary issues, importation of plasma samples to Belgium was not possible.

Still, we believe that this doctoral study can be considered a contribution to our knowledge on NTTAT and to attract the attention of the international research community, funding agencies and policy makers like the Ethiopian Ministry of Livestock and Fisheries. To the latter, we wish to pass the message that NTTAT control can only be successful when the epidemiological situation of the disease is known, when appropriate diagnostic tools and drugs are available and when intervention activities are undertaken on a regional level well beyond the national borders of a country.

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Curriculum vitae

Personal information

Name	Birhanu Hadush Abera
Sex	Male
Birth date	20 May 1980
Birth place	Mekelle, Tigray, Ethiopia
Marital status	Married
Number of children	Two
Nationality	Ethiopian
Language	Tigrigna, Amharic and English
Academic rank	Associate Professor
Contact address:	College of Veterinary Medicine, Mekelle University,Mekelle, Tigray, Ethiopia. P. O. Box 2084
E-mail	hadushbirhanu@yahoo.com

Educational background

Secondary school	Weldu Negusse Senior Secondary School, Mekelle, Tigray, Ethiopia, very great distinction (3.8/4), 1998.
University (DVM)	Addis Ababa University Faculty of Veterinary Medicine, Bishoftu, Oromia, Ethiopia, graduated as Doctor of Veterinary Medicine (DVM) with overall result of 2.68/4, 2004.
University (Masters)	Institute of Tropical Medicine, Antwerp, Belgium, graduated as Master of Science in Tropical Animal Health (Disease control) with overall result of 77%, in 2010.

Theses

2010: Use of Random Amplification of Polymorphic DNA (RAPD) for Identification of Potential Molecular Markers of *Trypanosoma (T.) brucei (b.) brucei*, *T. b. rhodesiense* and *T. b. gambiense*. (Partial fulfilment of Masters Degree in Tropical Animal Health, MSTAH).

2004: Equine Histoplasmosis: preliminary treatment trial, isolation and characterization of bacterial contaminants (Partial fulfilment of the degree of Doctor of Veterinary Medicine, DVM).

Other activities

October 2010 to March 2012	Head of Department of Veterinary Medicine, Mekelle University, Ethiopia.
January to August 2009	Leader of Veterinary Pathobiology and Infectious Diseases course team, Mekelle University, Ethiopia.
October 2007 to August 2008	Vice Dean for the then Faculty of Veterinary Science, Mekelle University, Ethiopia.
May 2006 to October 2007	Assistant Registrar for the then Faculty of Veterinary Sciences, Mekelle University, Ethiopia.
20 August 2004 to 13 April 2006	Head of animal health and farm manager Ethiopian Livestock Export Enterprise, Addis Ababa, Ethiopia.

Teaching

2015 to present Associate Professor at Mekelle University, College of Veterinary Medicine

2010 to 20 May 2015 Assistant Professor at Mekelle University, College of Veterinary Medicine. Courses: Veterinary Preventive Medicine, Infectious Diseases of Ruminants, Equines and Camels, Veterinary Entomology, Veterinary Protozoology.

2006 to 2009 Lecturer at Mekelle University, College of Veterinary Medicine. Courses: Veterinary Pathology, Veterinary Clinical Pathology, Veterinary Pathophysiology.

Publications in peer reviewed journals

1. **Birhanu, H**., Tadesse,G., Goddeeris, B.M., Büscher, P., Van Reet, N. 2016. New *Trypanosoma evans*i type B isolates from Ethiopian dromedary camels. *PLoS NTD* 10: e0004556.

2. **Birhanu, H.,** Fikru, R., Mussa, S., Weldu, K., Tadesse, G., Ashenafi, H., Tola, A., Tesfaye, D., Berkvens, D., Goddeeris, B.M., Büscher, P., 2015. Epidemiology of *Trypanosoma evansi* and *Trypanosoma vivax* in domestic animals from selected districts of Tigray and Afar regions, Northern Ethiopia. *Parasit. Vectors.* 8, 212.

3. **Birhanu, H.**, Roge, S., Simon, T., Baelmans, R., Gebrehiwot, T., Goddeeris, B.M., Büscher, P., 2015. Surra Sero K-SeT, a new immunochromatographic test for serodiagnosis of Trypanosoma evansi infection in domestic animals. *Vet. Parasitol*. 211, 153-157.

4. Jufare, A., Awol, N., Tadesse, F., Tsegaye, Y., **Hadush, B.**, 2015. Parasites of pigs in two farms with poor husbandry practices in Bishoftu, Ethiopia. *Onderstepoort J. Vet. Res*. 82, 839.

5. Kahsay, T., Negash, G., Hagos, Y., **Hadush, B.**, 2015. Pre-slaughter, slaughter and post-slaughter defects of skins and hides at the Sheba Tannery and Leather Industry, Tigray region, northern Ethiopia. *Onderstepoort J. Vet. Res*. 82, E1-E7.

6. Yimer, M.M., Bula, D.G., Tesama, T.K., Tadesse, K.A., **Abera, B.H.**, 2015. Prevalence of salivary gland hypertrophy syndrome in laboratory colonies and wild flies of *Glossina pallidipes* in Ethiopia. *Onderstepoort J. Vet. Res*. 82, 896.

7. **Birhanu H.**, Berihun A., Aregawi G., Nesibu A., Mulu A. and Kidane W. 2015: Preliminary Study on Mechanically Transmitted Bovine Trypanosomosis and Management of Trypanocidal Drugs in Selected Peasant Associations of Tigray, *Acta Parasitologica Globalis* 6 (1): 36-41.

8. Aboma R., Nesibu A., **Birhanu H.,** Yisehak T. and Teshale S. 2015: Internal and external parasites of camels (*Camelus dromedarius*) slaughtered at Addis Ababa Abattoir, Ethiopia, *JVMAH*. 7(2): 57-63.

9. **B. Hadush**, D. Biratu, H. Taddele, D.Tesfaye, G. Ameni 2014: Bacterial contaminants isolated from lesions of equine histoplasmosis in cart horses of Mekelle town, northern Ethiopia, *Revue Méd. Vét.*, 165 (1-2): 25-30.

10. Desalew T., Lisanework E., **Birhanu H**., Kassaw A. and Awot T., 2014: Study on the Efficacy of Selected Antitrematodal Drugs in Naturally Infected Sheep with Fasciolosis, *Acta Parasitologica Globalis* 5 (3): 210-213.

11. Erimiyas D., Berihun A., Etsay K., Nesibu A. and **Birhanu H.**, 2014: Seroprevalence of Trade Hampering Livestock Diseases in Animals Originated from Borana at Export Quarantine Centers in Adama, Central Ethiopia. *African Journal of Basic and Applied Sciences* 6 (2): 30-36.

12. Semere K., Nesibu A., Yisehak T. and Birhanu H., 2014: Hard Ticks of Camel in Southern Zone of Tigray, Northern Ethiopia*, J. Parasitol. Vector Biol*., 6(10): 151-155.

13. Nesibu A., Semere K., Yisehak T., Mohammed A., **Birhanu H**., 2014: Study on mange mite of camel in Raya-Azebo district, northern Ethiopia. *Veterinary Research Forum*. 5 (1) 61 – 64.

14. T T Gebrewahid, **B H Abera**, H T Menghistu, 2012: Prevalence and Etiology of Subclinical Mastitis in Small Ruminants of Tigray Regional State, North Ethiopia, *Vet. World*, 5(2): 103-109.

15. **Hadush B.**, Eshetu, L., Mengistu, W., Hailesilassie, M. 2009: Seroprevalence of contagious caprine pleuromonia in Kefta Humera, Alamata (Tigray) and Aba-'ala (Afar), Northern Ethiopia. *Trop. Anim. Health Prod*. 41: 803-806.

16. **Hadush, B.**, Kebede, E., Kidanu, H. 2008: Assessment of bacteriological quality of raw camels' milk in Ab-'Ala, North Eastern Ethiopia. *Livestock Research for Rural Development* 20, article #151, online at http://www.lrrd.org/lrrd20/9/hadu20151.htm.

17. **Hadush, B.**, Ameni, G., Medhin, G. 2008. Equine histoplasmosis: treatment trial in cart horses in Central Ethiopia. *Trop. Anim. Health Prod*. 40: 407-4011.

Conference presentations

1. **Birhanu, H**., Fikru, R., Mussa, S., Weldu, K., Tadesse, G., Ashenafi, H., Tola, A., Tesfaye, D., Berkvens, D., Goddeeris, B.M., Büscher, P., 2015. Epidemiology of *Trypanosoma evansi* and *Trypanosoma vivax* in domestic animals from selected districts of Tigray and Afar regions, Northern Ethiopia. 33rd Meeting of International Scientific Council for Trypanosomosis Research and Control (ISCTRC), September 14-18, Ndjamena, Chad.

2. **Birhanu, H.**, Roge, S., Simon, T., Baelmans, R., Gebrehiwot, T., Goddeeris, B.M., Büscher, P., 2015. Surra Sero *K*-SeT, a new immunochromatographic test for serodiagnosis of *Trypanosoma evansi* infection in domestic animals. 33rd Meeting of International Scientific Council for Trypanosomosis Research and Control (ISCTRC), September 14-18, Ndjamena, Chad.

3. **Birhanu, H**., Fikru, R., Mussa, S., Weldu, K., Tadesse, G., Ashenafi, H., Tola, A., Tesfaye, D., Berkvens, D., Goddeeris, B.M., Buscher, P., 2015. Epidemiology of *Trypanosoma evansi* and *Trypanosoma vivax* in domestic animals from selected districts of Tigray and Afar regions, Northern Ethiopia. The 3rd International Veterinary Education Conference (IVEC), June 17-19, Haromaya University, Harrar, Ethiopia.

4. **Birhanu, H.**, Gebrehiwot T., Goddeeris, B. M., Van Reet ,N., Büscher, P. 2015. *Trypanosoma evansi* type A and type B strains from Tigray and Afar regions of Northern Ethiopia. Non Tsetse transmitted Animal Trypanosomosis (NTTAT), OIE adhoc committee, 24 May, Paris, France.

5. **Birhanu H**. 2015. Information Communication Technologies (ICTs) use in Education and Animal Husbandry (review). The 3rd International Veterinary Education Conference (IVEC), June 17-19, Haromaya University, Harrar, Ethiopia.

6. **Birhanu, H**. 2014. Potential impacts of climate change on public and animal health in sub-Saharan Africa (review). International conference on enhancing economic growth and strengthening public health through livestock development and one health approach, May 8-10, College of Veterinary Medicine, Mekelle University, Mekelle, Tigray, Ethiopia, pp 45.

7. **Birhanu H**. 2012. Impact of climate change on human and animal health in Africa (review). Inaugural conference of Ethiopian Society of Tropical and Infectious Diseases (ESTAIDs), December 7-8/2012; Economic Commission for Africa (UN- ECA) conference hall, Addis Ababa, Ethiopia.

8. **Birhanu H**. 2012. Opportunities and challenges for implementation of one health approach in sub-Saharan Africa (SSA) (review). International ITM 5th Alumni Riprosat, One Health, One World, the context of developing countries: Opportunities and challenges, October 1-3, Addis Ababa Ethiopia.

9. **Birhanu H**., Etsay K., Hailay, K. 2012. Assessment of bacterial quality of raw camels' milk in Ab-'Ala, North Eastern Ethiopia. International ITM 5th Alumni Riprosat, One Health, One World, the context of developing countries: Opportunities and challenges, October 1-3, Addis Ababa Ethiopia.

10. Assefa T., Tamrat, H., Gobena, A., Verma,P.C., Abreha,T., and **Birhanu H**., 2012. Epidemiology of Bovine Tubercullosis in cattle slaughtered in municipal abbttoirs of Mekelle and Nekemte. 7th National TB research conference, fffective partnership to combat TB: Rallying the forces in Ethiopia, March 21-23, Mekelle University and Tigray Bureau of Health, Mekelle, Tigray, Ethiopia, Abstract number 12, pp. 15.

10. **Birhanu H**., Etsay K., Hailay, K. 2011. Assessment of bacterial quality of raw camels' milk in Ab-'Ala, North Eastern Ethiopia. XVth International Society for Animal Hygiene Congress on animal hygiene and sustainable livestock production, 3-7 July 2011, Vienna, Austria.

11. **Birhanu H**., Lisanework, E., Wubshet,M., Mekonnen, H. 2011. Seroprevalence of contagious caprine pleuropneumonia in Tigray and Afar, Northern Ethiopia. XVth International Society for Animal Hygiene Congress on animal hygiene and sustainable livestock production, 3-7 July 2011, Vienna, Austria.

12. **Birhanu H**., Etsay K., Hailay, K. 2011. Assessment of bacteriological quality of raw camel's milk in Ab-'Ala, North Eastern Ethiopia. ITM-DVTD International Colloquium on Zoonoses and Neglected Infectious Diseases of Africa. 1-4 November 2011, Pretoria, South Africa, Abstract number 88, pp. 49.

13. **Birhanu H**., Delespaux, V., Büscher, P., Rogé, S., Balharbi, F., Deborggraeve, S. 2011. Use of random amplified polymorphic DNA (RAPD) analysis for the identification of potential molecular markers for *T.b. brucei, T.b. gambiense* and *T.b. rhodesiense*. ITM-DVTD International Colloquium on Zoonoses and Neglected Infectious Diseases of Africa. 1-4 November 2011, Pretoria, South Africa, Abstract number 89, pp. 50.

Short term trainings

26 October 2015	Genetic engineering, Gent
October 2014 to February 2015	Advanced and Applied Molecular Biology, Vrjie Universiteit Brussel (VUB), Brussels, Belgium (formal training) (5 ECTs).
5 to 9 January 2015	Introduction to basic Geographical Information System (GIS), Institute of Tropical Medicine, Antwerp, Belgium (3 ECTs).
28 July to 8 August 2014	Global challenges: Urbanization, livelihoods and food security, PhD course, Gaborone, Botswana (10 ECTs).
31 January, 3 and 14 February 2013	Bioinformatics at VIB. Basic Bioinformatics, concepts, databases and tools, Leuven, Belgium.
2 to 3 December 2013	How to write a winning grant proposal' of training@VIB, KU Leuven, Leuven, Belgium.
16 to 20 September 2013	FLAMES summer school training on Methodology and Statistics, KU Leuven, Belgium.
20 September 2012	Pipetting with Ranin, Institute of Tropical Medicine, Antwerp,Belgium.
19 September 2012	Seminar of inflammation and vaccination, University of Ghent, Belgium.
29 to 31 August 2012	Specialist course 'Workshop Model Organisms: Nature's gift to translational research' Flemish Training Network in Life Sciences (FTNLs) University of Hasselt, Belgium (3 ECTS).
05 to 16 March 2012	Climate change and natural resources management, Wageningen UR Centre for Development Innovation, Addis Ababa, Ethiopia.
23 to 25 August 2011	Health Research Ethics US Department of Health and Human Services and Tigray Science and Technology Agency, Mekelle, Tigray, Ethiopia.
12 June to 13 July 2006	Serological techniques, National Veterinary Institute, Bishoftu, Oromia, Ethiopia.